

Textbook of Assisted Reproductive Techniques

Sixth Edition

Volume 1: Laboratory Perspectives



Edited by
DAVID K. GARDNER
ARIEL WEISSMAN
COLIN M. HOWLES
ZEEV SHOHAM

Textbook of Assisted Reproductive Techniques

Established as the definitive reference for the IVF clinic, this Sixth Edition has been extensively revised, with the addition of several important new contributions on laboratory topics, including KPIs for the IVF laboratory, quality control in the cloud, artificial intelligence, AI in gamete and embryo selection, demystifying vitrification, microfluidics, gene editing, disaster management, enhanced imaging of early embryo development, and artificial gametes. As previously, methods, protocols, and techniques of choice are presented by IVF pioneers and eminent international experts.

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PREFACE

The first edition of the *Textbook of Assisted Reproductive Techniques* was published in 2001. As the textbook now enters its sixth edition, some 45 years since the birth of Louise Brown, the world's first test tube baby in the United Kingdom, it is remarkable to reflect upon the changes in assisted human conception that have been documented in each successive edition of the textbook.

Over the past 20 years, we have witnessed the widespread implementation of single blastocyst transfer, and the ability to undertake trophectoderm biopsy and genetic analysis now using next-generation sequencing to accurately determine chromosomal copy number, and to provide precise genetic diagnosis for patients as needed. This shift in practice of transferring only one high-quality embryo has brought us closer to the mantra of "one embryo, one baby." Cryopreservation, historically performed using slow-rate controlled freezing, has now been superseded by vitrification for both oocytes and embryos, with oocyte cryopreservation becoming a realistic treatment for fertility preservation, especially for oncology patients and younger women wishing to preserve their fertility. Improvements in laboratory culture techniques and incubation devices, including time-lapse imaging, have also contributed to the adoption of single embryo transfers without reducing the chance of a live birth. Excitingly, more technologies are now available for sperm assessment, and the knowledge underpinning *in vitro* maturation has facilitated the development of potential new approaches for IVF.

As for ovarian stimulation protocols, there has been, over the past 20 years of this textbook series, a major shift in practice. The clinical acceptance of the GnRH antagonist protocol, first registered in 1999, took more than 10 years to be widely adopted. With the possibility of using a GnRH agonist to trigger follicular

maturity, the protocol has become the preferred choice, facilitating the concept of an "OHSS-free clinic." A plethora of new pharmaceutical FSH agents have been introduced into practice that have resulted in increased patient convenience and drug delivery precision (due to the use of pen devices) rather than increased live birth rates. This is a further reflection of the complexity of the overall IVF treatment process—in particular, the pivotal role that the embryology laboratory continues to play in improving cycle success.

Sadly, however, over the duration of this textbook's life span, we have lost several authors—all dear friends and colleagues—whom we miss and to whom we are grateful for their enormous contributions to our field during their lifetimes:

- Marinko Biljan, Quebec
- Isaac Blickstein, Rehovot
- Jean Cohen, Paris
- Howard W Jones Jr, Norfolk
- Michelle Lane, Adelaide
- Ragaa Mansour, Egypt
- Queenie V Neri, New York
- Lynette Scott, Boston
- Carl Wood, Melbourne
- Yury Velinsky, Chicago

Finally, we lost one of the pioneering fathers of this field, Bob Edwards, a giant in our field on whose shoulders we have all been fortunate to stand.

**David K. Gardner, Ariel Weissman,
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UPDATED GUIDELINES FOR SETTING UP AN ASSISTED REPRODUCTIVE TECHNOLOGY LABORATORY

Jacques Cohen, Mina Alikani, and Antonia Gilligan

There are many ways to set up and operate assisted reproductive technology (ART) laboratories; one set-up may have little in common with another but prove to be equally successful. This is important to remember as one ventures into establishing a new clinic or open-laboratory ART system. Facilities for ART range from a makeshift *in vitro* fertilization (IVF) laboratory with a minimum of equipment to a fully equipped laboratory specifically designed for ART, sometimes with additional space dedicated to clinical training and research. There have been major changes in IVF and ART laboratories during the last decade with the introduction of robotics of embryo culture and cryopreservation of all embryos. More such changes are expected soon. The change to robotics of micromanipulation procedures such as intracytoplasmic sperm injection (ICSI), cryopreservation, cryo-storage, and gamete and embryo handling is requiring a change to highly technical and ever more specialized space. Some spaces may reduce in size because of automation and miniaturization. Other spaces such as cryo-storage are likely to increase due to the growing number of samples along with the potential use of relatively large robots for automated tracking during cryo-storage. This chapter does not cover gamete retrieval laboratories, which may incorporate retrieval and transport of gametes and embryos to other locations. Although such models can be successful, compelling evidence showing that they produce optimal results is still lacking [1, 2]. IVF, ICSI, and oocyte cryopreservation can be applied to transported oocytes, and in certain situations “transport IVF” is a welcome alternative for those patients whose reproductive options have been limited by restrictive governmental regulations [2, 3]. This chapter discusses the more typical purpose-built, all-inclusive laboratories that are adjacent or in close proximity to oocyte retrieval, cryo-storage, and embryo transfer facilities, with an emphasis on the special problems of construction. For choices of culture system, culture medium, supplementation, viability assays, handling and processing of gametes and embryos including freezing and vitrification, and cryo-storage, the reader is referred to other relevant chapters in this textbook. An international expert meeting on the construction, technical, and operational requirements for ART laboratory air quality established 50 consensus points regarding site suitability, design criteria for new construction, and laboratory commissioning [4]. This consensus meeting has provided standards for existing laboratories and guidelines for constructing new laboratories. The Cairo consensus meeting also proposed guidelines regarding current laboratory practice such as how to decrease volatile organic compounds in incubators and in the ambient space.

Personnel and experience

Although the environment, physical plant, and equipment require special consideration in the design of an integrated gamete and embryo culture facility, the staff will carry out the procedures

and therefore is essential to the success of the entire operation. Successful clinical practice, in general, and ART, in particular, are almost entirely dependent on the skill and experience level of medical and laboratory personnel. Some planners look for a facility and location first, but staffing and caseload must be considered early on in the process. For the laboratory staff, enthusiasm is another key factor to success, especially because there are still few formal teaching and skills examination programs in place for a specialty in ART. Most clinical embryologists are trained using an apprenticeship program, but such institutions are rare and there are no internationally accepted guidelines. Non-apprentice hands-on training facilities are now offered in several locations in the USA, with one-on-one training options ranging from weeks to several months. This has been a huge step forward in assisting clinics to reduce or facilitate traditional in-house training. Good clinical outcome requires a cautious and rational assessment of individual abilities, so laboratory staff, directors, and embryologists must consider their experience in the context of what will be required of them [5, 6].

This chapter aims to provide information necessary for experienced practitioners to set up a new laboratory. Setting up a new laboratory or thoroughly renovating an existing facility is very much an art, as is the practice of ART itself. We do not recommend that new laboratories and ART clinics are built by administrators, engineers, or architects without considerable input from experienced embryologists, technical staff, and clinicians. Another consideration is to have input from existing laboratory staff rather than let outside consultants make all the decisions.

Programs should develop a system of tracking individual performances for crucial clinical and laboratory procedures such as embryo transfer efficiency, ICSI, and biopsy proficiency, among others. This is easily achievable using a conventional performance tracking system or digital record-keeping. Certain regulatory bodies such as the College of American Pathologists (CAP) in the USA and the Human Fertilisation and Embryology Authority (HFEA) in the UK provide guidelines and licensing for embryologists, sometimes even for subspecialties such as the performance of ICSI, the practice of embryo biopsy, and directing IVF and andrology laboratories. So far, such licensing has done little more than increase workload because licensing does not necessarily guarantee skill (or success) and the licenses may not be valid across borders.

Tradition also plays its role. For example, in some Asian countries, embryology directors are usually medical professionals. Thus, academic titles are often seen as being more important than actual qualifications. What then qualifies someone to be a laboratory director or an embryologist? The answer is not simple. In general, current licensing authorities, including the American Board of Bioanalysis (ABB), consider individuals trained in general pathology or reproductive medicine and holding an MD degree along with individuals holding a PhD degree qualified to

be laboratory directors if they meet some other requirements. However, pathologists do not necessarily have experience in gamete and embryo cell culture, and some reproductive medicine specialists, such as urologists and immunologists, may have never worked with gametes and embryos. It is possible for a medical practitioner to direct a laboratory in certain countries without ever having practiced gamete and embryo handling! *Eppur si muove* ("And yet it moves"), as Galileo said when condemned by the Roman inquisition for the heresy of accepting Copernican astronomy. Once there are rules, even silly ones, it can be hard to change them.

Empirical and statistical requirements for staff

There is considerable disagreement about what should be the required experience for embryologists. Hands-on experience in all facets of clinical embryology is an absolute condition when starting a new program. Even highly experienced experimental embryologists and animal scientists should be directly supervised by experienced clinical personnel. The period during which close supervision must continue depends on the types of skills required, the daily caseload, and time spent performing procedures. Clearly, performing 100 cases over a one-year period is a very different circumstance than performing the same number over six weeks; the period of supervision then should be adjusted accordingly. Experience is not just dependent on caseload but also on egg yield, as workload is proportional to type and extent of follicular stimulation protocols.

The optimal ratio of laboratory staff to the expected number of procedures is debatable, and, unfortunately, economics play an all too important role here. However, with the incorporation of new technologies and treatment modalities in routine care, the complexity of IVF laboratory operations has increased substantially over the past decade, in turn requiring more careful consideration of staffing levels [6]. According to some calculations, whereas a "traditional" IVF cycle required roughly 9 personnel hours, a contemporary cycle can require up to 20 hours for completion. Thus, the number of embryologists required for safe and efficient operation of the laboratory has also increased. Based on a comprehensive analysis of laboratory tasks and their complexity, an Interactive Personnel Calculator was introduced nearly 10 years ago to help laboratory directors and administrators determine staffing needs [6]. This calculator may benefit from updating since the overall level of activity is determined by a clinic's quality assurance program, culture methodology, the average number of oocytes retrieved, and the incidence of cryo-storage of both gametes and embryos. ART laboratories have transitioned to cryo-storage facilities over the years, but automation and continuous digital tracking of samples are still not implemented in spite of evident advantages and the availability of at least one FDA-approved robotic system (TMRW, USA). Overall, it is safe to say that the ratio of laboratory staff to caseload should be high enough so that embryologists can not only safely perform procedures but also dedicate time to quality control and continued education and training to maintain the high standards required for success. Staff burnout is not just a function of the quantity of procedures but also of the quality of staff interactions and high-intensity aspects of assisted reproduction. The challenge of keeping these standards within national health systems or in the face of insurance mandates that must provide a wide range of services on a minimal budget is real but should not be insurmountable. Patients usually

do not benefit from such constraints, as a comparison of results in different health service systems in Western countries would suggest. There are limitations to such comparisons, but live births per embryo and cumulative data from fresh and cryopreservation cycles are considered objective assessments [7].

The job description for the embryologist ideally includes all embryology and andrology tasks, except for medical and surgical procedures. Embryologists are often involved in other important tasks as well, including patient management, follicular monitoring, genetic counselling, marketing, running diagnostic laboratories, and administration. It should be realized that these tasks may detract from their main responsibilities. First and foremost, the duty of an embryologist is to safely perform gamete and embryo handling and culture procedures. Second, but equally important, the embryologist should maintain quality control standards, both by performing routine checks and tests and by maintaining detailed logs of incidents, changes, unexpected events, and corrective measures. Across all these duties, the following seven positions can be clearly defined: director, supervisor, senior embryologist, embryologist, trainee, assistant, and technician. There may also be positions for others to do preimplantation genetic testing and research; to validate new procedures; and for quality control supervision, technical supervision, and administrative work. Obviously, not all of these separate positions are necessary for smaller centres, and tasks can be combined.

Although a seemingly unimportant detail, one of the most important jobs in the IVF laboratory at Bourn Hall Clinic in Cambridge, UK, during the first few years of operation was that of a professional witness and embryology assistant. This position was the brainchild of Jean Purdy, the third partner with Patrick Steptoe and Robert Edwards, who was involved in the work that led to the birth of Louise Brown. The embryology assistant effectively enforced and oversaw the integrity of the chain of custody of gametes and embryos during handling, particularly when large numbers of patients were being treated simultaneously. The "witness" also ensured that embryologists performed only those procedures for which they were qualified. Interestingly, recent literature suggests that this crucial concept has not been universally and fully understood or adopted by all IVF laboratories. In one group of laboratories [8], "limited and consequently virtually ineffective" witnessing processes were only abandoned in favor of a more robust witnessing program after implementation of a failure mode and effects analysis (FMEA) showed a high risk of error in gamete and embryo identification. The authors stated that, "Only after FMEA optimization has the witness embryologist been formally recognized as a committed role, specifically trained for witnessing shift work." Hopefully, this and other similar studies [9] that show the effectiveness of a witnessing system will encourage more laboratories to re-examine their practices and allocate adequate resources to ensuring the safety and efficiency of all procedures performed by the laboratory.

Facility, design, and budget

In the early days of IVF some clinics were built in remote areas, based on the premise that environmental factors such as stress could affect the patient and thereby the outcome of treatment. Today's laboratories are commonly placed in city centres and large metropolitan areas in order to service large populations locally. It is important that patients understand that there have been millions of others like them before and that, in general,

IVF is a routine, though complex, medical procedure. It is clear that the choice of a laboratory site is of great importance for a new program. The recent development of better assays for determining the baseline quality of the environment facilitates site selection. There is now awareness that some buildings or building sites could be intrinsically harmful to cell tissue culture [10–13]. The direct effect of poor air quality and the presence of volatile organic compounds (VOCs) on IVF outcomes has been demonstrated by recent studies of countermeasures such as novel filtration systems and an understanding of the partitioning of compounds across liquid phases such as water and oil [4, 12, 14]. A laboratory design should be based on the anticipated caseload and any subspecialty. Local building and practice permits must be assessed prior to engaging and completing a design. There are five basic types of design:

1. Laboratories using only transport IVF
2. Laboratories adjacent to clinical outpatient facilities that are only used part of the time
3. Full-time clinics with intra-facility egg transport using portable warming chambers
4. Fully integrated laboratories with clinical areas
5. Moveable temporary laboratories

Before developing the basic design for a new laboratory, environmental factors must be considered. Although air quality in modern laboratories can be controlled to a degree, it can never be fully protected from the exterior environment and adjoining building spaces. Designers should first determine if the building or the surrounding site is scheduled to undergo renovations, demolition, or major changes of any kind in the foreseeable future. City planning should also be reviewed. Historical environmental data and trends, future construction, and the ability of maintenance staff to maintain and service the IVF laboratory need to be determined. Activity related to any type of construction can have a significant negative impact on any proposed laboratory. Prevalent wind direction, industrial hazards, and general pollution reports such as ozone measurements should also be determined. Even when these factors are all deemed acceptable, basic air sampling and determination of VOC concentrations is necessary inside and outside the proposed building area. IVF laboratory VOC concentrations have traditionally involved determination in parts per billion (ppb) when evaluating individual compounds such as deleterious aldehydes. This has required determination by laborious methods such as gas chromatography and mass spectrometry, but more recently total VOC counts have become a way of assessing laboratory conditions, allowing the introduction of affordable handheld units that measure in parts per million (ppm). At least one easy to use cloud-based handheld unit exists (Graywolf Sensing Solutions, USA) that can determine common VOCs in ppb in real time, although it is relatively expensive. The outcome of initial space tests will determine which design requirements are needed to remove VOCs from the laboratory area. In most cases, an over pressured laboratory (at least 0.10–0.20 inches of water) that uses a high number [7–14] of fresh air changes per hour is the best solution, because it also provides for proper medical hygiene. The Cairo consensus [4] has set important standards for this crucial element of laboratory design and maintenance. It was recommended that total VOCs be maintained less than $500 \mu\text{g}/\text{m}^3$ (~ 400 – 800 ppb total VOC, depending on molecular species); less than $5 \mu\text{g}/\text{m}^3$ aldehydes. Experience has shown that aldehydes can be very toxic to IVF

conditions even at low levels. Few handheld devices can quantify this group of chemicals accurately.

The laboratory walls and ceiling should have the absolute minimum number of penetrations. This generally requires a solid ceiling, sealed lighting, and airtight utility connections. Contrary to many vendors' representations, commercial suspended ceilings using double-sided tape and clips are not ideal. Doors will require seals and sweeps and should be lockable. Ducts and equipment must be laid out in such a way that routine and emergency maintenance and repair work can be performed outside the laboratory with minimal disruption to the laboratory. Air handling is not optimal when using an open plenum design. In the ideal case, 100% outside air with chemical and physical filtration will be used with sealed supply and return ducts.

While providing cleaner air, 100% outside air sourcing will maximize the life of a chemical filter and will provide a lower concentration of VOCs in the IVF laboratory's air. In climates where temperatures routinely exceed 32°C with 85%-plus relative humidity, 100% outside air could result in an unacceptable level of humidity (>60%), which could allow mould growth. In these cases, the use of limited return air from the lab is acceptable. A 50% outside air system with 15–30 total air changes per hour to maintain over pressure works well and the relative humidity becomes very controllable. To place this in perspective, traditional medical operating room design calls for 10%–15% outside air.

The air supply equipment may supplement outside air with recirculated air, with processing to control the known levels of VOCs. On rare occasions, laboratories will require full-time air recirculation, although most may actually find the outside air to be perfectly clean at least most of the time. Outside air is often erroneously judged to be polluted without proper chemical analysis, whereas inside air is usually considered "cleaner" because it may "smell" better. In most laboratory locations, conditions are actually the reverse, and designers should not "follow their instincts" in these matters. Humidity must also be completely controlled according to climate and seasonal variation. The system must be capable of supplying the space with air with a temperature as high as 30°C – 35°C at less than 40% relative humidity. Air inlets and outlets should be carefully spaced to avoid drafts that can change local "spot" temperatures or expose certain equipment to relatively poor air or changes in air quality. Laminar flow hoods and micromanipulation workstations should not be located too close to air supply fixtures to avoid disruption of the sterile field and to minimize cooling on the microscope stage. Semi-enclosed workstations based on Class 2 cabinets or neonatal isolette incubators can be considered to optimize the work environment and bridge the gap between the incubator and the workstation. A detailed layout and assessment of all laboratory furniture and equipment is therefore essential prior to construction and has many other benefits.

Selection of an experienced and subspecialized (and flexible) architect and a mechanical engineer for the project is essential. Confirm what their past experience has been in building biologically clean rooms. The use of "environmentally friendly" or "green" products has been suggested by some designers. The reliance on "natural" products does not ensure a clean laboratory. In one case, wood casework with a green label was found to be a major source of formaldehyde. Floor coverings using recycled vinyl and rubber were selected for their low environmental impact, without considering the significant release of trapped gases by the material.

Supervision of the construction is also critical. Skilled tradespersons using past training and experience may not follow all the architect's instructions. The general contractor and builders must be briefed on why these novel construction techniques are being used. They must understand that the use of untested methods and products can compromise the project (and the payment of their fees!). Contractual agreement is recommended. An initial operations and materials discussion with construction crews is highly recommended along with frequent inspections by a senior embryologist and informed architect.

Just as the organization and flow of traffic in a world-class restaurant result in a special ambience where more than just the food is the attraction, appropriate modular placement of equipment ensures safety and comfort in the over pressured IVF laboratory. Placement of stacks of incubators, gamete handling areas (laminar flow units or isolettes), and micromanipulation stations should minimize distances that dishes and tubes need be moved. Ideally, an embryologist should be able to finish one complete procedure without moving more than three meters in any direction; not only is this efficient, but also it minimizes accidents in a busy laboratory. Design and implementation of a work area incorporating product, gas and liquid nitrogen supplies, and a workstation, refrigerator, and incubators is feasible even without the embryologists having to walk between storage cabinets and equipment. Such a modular design can be duplicated multiple times within a larger air handling area allowing the handling of large numbers of gametes and embryos. For logistical reasons, sperm preparation and cryopreservation may be placed in adjacent areas. The number of modules can easily be determined by the expected number of cases and procedure types, the average number of eggs collected, and the number of embryologists expected to work simultaneously. Each person should be provided with sufficient workspace to perform all procedures without delay. Additional areas can contain simple gamete handling stations or areas for concentrating incubators. Cryopreservation and storage facilities are often located in separate spaces; if separated, these areas should always be adjacent to the main laboratory. Storage spaces could be separated further using closets or rooms with negative pressure. Embryology laboratories have undergone tremendous changes recently, with the advent of successful cryopreservation through vitrification. Vitrification allows replacement in natural cycles on a routine basis. What used to be a small room is now becoming a sizable cryo-storage facility, even though offsite storage location is recommended for samples with unclear future dispositions. The need for a separate area for ancillary activities such as medium preparation is clearly diminishing now that commercial manufacturers provide all the basic needs of an IVF laboratory. Administration should probably be performed in separate offices on a different air handling system from the main laboratories, though prospective studies regarding the effect of actual paperwork on outcomes do not exist.

Last but not least, it is preferable to prepare semen in a separate laboratory altogether, adjacent to one or more collection rooms. The semen laboratory should have ample space for microscopes, freezing, and sterile zoning. Proper separation of patient samples during processing is essential, and some elemental design features accommodating this may be considered before the first procedures are carried out. Some thought should go into planning the semen collection area. This small room should be at the end of a hallway preferably with its own exit; it should be soundproof, not too large, with a sink, and under negative pressure if possible. Clear instructions on how to collect semen for ART should be

provided in the room. The room should also be adjacent to the semen preparation laboratory, preferably with a double-door pass-through for samples. This pass-through should have a signalling device so the patient can inform the embryologist that the sample is ready; it also permits male patients to leave the area without having to carry a specimen container.

Equipment and storage

A detailed list of equipment should be prepared and checked against the planned location of each item; it can later be used as the basis of maintenance logs. It is important to consider the inclusion of crucial equipment and spare instruments in the laboratory design to allow for unexpected malfunction. Similarly, two or more spare incubators should not be seen as excessive; at least one spare follicle aspiration pump and micromanipulation station (equipped with a laser) should also be included. The use of a spare liquid nitrogen-primed Dewar is now mandatory in some countries. There are many other instruments and equipment pieces the malfunction of which would jeopardize patient care, although some spares need not be kept on hand as manufacturers may have them available; however, such details need to be repeatedly checked as suppliers' stocks continue to change. It may also be useful to team up with other programs or an embryology research laboratory locally so that a crucial piece of equipment can be exchanged in case of unexpected failure.

Some serious thought is needed when contemplating the number and type of incubators (for a comprehensive review, see [15]). The ratio of incubators to patient procedures depends on incubator size and capacity and it varies considerably from program to program. It is clear that the number and type of incubator, along with the length and number of incubator door openings, affect results. In principle, the number of cases per incubator should be kept to a minimum. The smaller box incubators should not handle more than two to three cases. In benchtop incubators, the use of one dish slot per patient is not recommended. Dishes for one patient should be kept in one compartment, preferably with its own lid door. Several other incubators can be used for general purposes during micromanipulation and for other generic uses to limit further the number of incubator openings. Strict guidelines must be implemented and adhered to when maintaining distinct spaces for separating culture dishes or tubes of different patients. Tracking of incubators and even shelves or compartment spaces within each incubator is recommended so their performance can be evaluated on an ongoing basis. Separate compartments within an incubator may be helpful and can be supplied by certain manufacturers. Servicing and cleaning of equipment such as incubators may have to be done when the laboratory is not performing procedures. Placement of incubators and other pieces of equipment on castors may be helpful in programs where downtime is rare. Pieces of equipment can then be serviced outside the laboratory. New incubators and equipment pieces that come in contact with gametes and embryos must be "burned in" or "off-gassed." Protocols vary per equipment type and manufacturer.

When there are several options available to the laboratory designer, supply and evacuation routes should be planned in advance. One of the most susceptible aspects of ART is cryopreservation. In case of an emergency such as a fire or power failure, it may be necessary to relocate the liquid nitrogen-filled Dewars without using an elevator, or to relocate the frozen samples using a temporary container. This may seem an extreme consideration, especially in the larger laboratories that stockpile

thousands of samples, but plans should be made. It may be possible to keep a separate storage closet or space near the building exit where long-term samples, which usually provide the bulk of the storage, can be kept; this would require repeated checking of a facility that is not part of the laboratory. Liquid nitrogen tank alarms with remote notification capability should be installed on all Dewars holding gametes and embryos. The route of delivery of liquid nitrogen and other gas cylinders must be relatively easy, without stairways between the laboratory and the delivery truck, and should be sensibly planned. Note that the flooring of this route is usually destroyed within months because of liquid nitrogen spills and wear caused by delivery containers, so the possibility of an alternative delivery corridor should be considered for these units.

Liquid nitrogen containers and medical gas cylinders are preferentially placed immediately adjacent to the laboratory in a closet or small, ventilated room with outside access. Pipes and tubes enter the laboratory from this room, and cylinders can be delivered to this room without compromising the laboratory area in any way. Providing liquid nitrogen and even liquid oxygen vapor to triple gas incubators is nowadays a preferred option since vapor is cleaner than compressed gas. This allows liquid nitrogen vapor to be pumped into the cryopreservation laboratory using a manifold system and minimal piping. Lines should be properly installed and insulated to ensure that they do not leak or allow condensation and conserve energy at the same time. Medical gases can be directed into the laboratory using pre-washed vinyl/Teflon-lined tubing such as fluorinated ethylene propylene, which has high humidity, temperature, and UV radiation stability. Lines should be properly marked every meter indicating the incubators supplied in order to facilitate later maintenance. Alternatively, solid manifolds made from stainless steel with suitable compression fittings can be used. Avoid the soldered or brazed copper lines used in domestic plumbing applications wherever possible; copper lining can be used but should be cleaned and purged for a prolonged period prior to use in the laboratory. Copper line connections should not be soldered as this could cause continuous contamination. This recommendation may conflict with existing building codes, but non-contaminating alternatives can be found. A number of spare lines or conduits hidden behind walls and ceilings should be installed as well, in case of later renovation or facility expansion.

Large programs should consider the use of exterior bulk tanks for carbon dioxide and liquid nitrogen. This removes the issue of tanks for incubators or cryopreservation. These tanks are located where delivery trucks can hook onto and deliver directly to the tank. Pressurized gas lines or cryogenic lines then run the carbon dioxide or liquid nitrogen to the IVF laboratory for use.

Placement of bulky and difficult pieces of equipment should be considered when designing doorways and electrical panels. Architects should be fully informed of all equipment specifications to avoid the truly classic door width and height mistakes. Emergency generators should always be installed, even where power supplies are usually reliable. The requirements can be determined by an electrical engineer. Thankfully, these units can be removed from the laboratory but must be placed in well-ventilated areas that are not prone to flooding. Additional battery “uninterruptible power systems” may be considered as well, but may be of limited capability and costly. Buildings should also be checked for placement of the main power inlets and distribution centres, especially because sharing power lines with other departments or companies may not be advisable. Circuit breakers

should be easily accessible to embryologists or building maintenance staff. General knowledge of the mechanical and electrical engineering of the building and the laboratory specifically will always be advantageous. Leaving all the building mechanics and facilities to other individuals is often counterproductive. Embryologists need to be involved with facilities management and be updated with construction decisions inside and outside the building in a timely manner.

Ample storage spaces should always be planned for IVF laboratories. In the absence of dedicated storage space, laboratory space ends up being used instead, filling all cabinets and negating any advantages of the original design. The dedicated storage area should be used to stock all materials in sufficient quantity to maintain a steady supply. A further reason to include storage areas in laboratory design—sufficient on its own to justify the space—is that new supplies, including sterile disposable items, release multiple compounds for prolonged periods. This “out-gassing” has been determined to be a major cause of air pollution in a number of laboratories in which supplies were stored inside the lab. Separate storage space therefore provides the best chance of good air quality, especially when it is supplied by separate air handling system and under negative pressure. It should be large enough to handle bulky items and mobile shelving for boxes. One should be careful to avoid the natural inclination to save extra trips by bringing too many items into the laboratory, or the gains made by careful design may be lost. As a possible makeshift solution, storage cabinetry in the laboratory can be designed with separate negative pressure air handling to minimize release of VOCs from off-gassing package materials.

Microscopes and visualization of cells

Though dissecting microscopes are crucial for the general handling of gametes and embryos, many people still consider inverted microscopes to be a luxury even though they are in regular use with micromanipulation systems. Proper visualization of embryos is key to successful embryo selection for transfer or freezing; if the equipment is first class, visualization can be done quickly and accurately [16]. Even so, appropriately detailed assessment still depends on the use of an oil overlay system to prevent damage by prolonged exposure. Each workstation and microscope should be equipped with a still camera and/or video camera and monitor. Still photos can be placed in the patient file, and video footage permits speedy review of embryonic features with colleagues after the gametes are safely returned to the incubator; this is also helpful for training new embryologists. Recordings can be uploaded onto a patient health information, secure cloud service or kept on secure servers in the facility. Interference optics such as Hoffman and Nomarski are preferable because they permit the best measure of detail and depth. Novel visualization of internal elements such as spindles using polarized microscopy requires additional equipment but can be incorporated into routine operation [18]. Ideally, the captured photos should be digitally stored for recall in the clinic’s medical database.

Development of new time-lapse microscopy technologies has made continuous and uninterrupted monitoring of embryo development a reality. This is an invaluable teaching and learning tool. However, equipment costs are high and, for many laboratories, prohibitive. Equipment for time-lapse technology can be sizable and may require separate consideration in terms of lab design and bench space.

Construction, renovation, and building materials

Construction and renovation can introduce a variety of compounds into the environment of the ART laboratory, either temporarily or permanently. Either can have major adverse effects on the outcome of operations [10–12, 18, 19]. The impact of the exterior environment on IVF success has been demonstrated. Pollutants can have a significant negative effect on success in an IVF laboratory [10, 20]. These effects can range from delayed or abnormal embryonic development, reduced or failed fertilization, and reduced implantation rates to pregnancy loss and failure of a treatment cycle. Many of the damaging materials are organic chemicals that are released or outgassed by paint, adhesives from flooring, cabinets, and general building materials, along with laboratory equipment and procedures. It is important to realize that the actual construction phase of the laboratory can cause permanent problems. Furthermore, any subsequent renovation activity in adjacent areas can also cause similar or even greater problems. Neighbouring tenants can be informed of the sensitivity of gametes and embryos in culture. At the very least, changes undertaken in adjacent areas should be supervised by IVF laboratory personnel to minimize potential damage. However, new construction immediately outside the building is considerably more problematic. City works such as street construction are very hard to predict and nearly impossible to control. A good relationship with the neighbours should be maintained and a working relationship with building owners and city planners should be established so that the IVF laboratory is kept informed of upcoming changes.

For the construction of a new laboratory or if changes are to be made to areas adjacent to the IVF facility, the following guidelines should be followed. First, the area to be demolished and reconstructed needs to be physically isolated from the IVF laboratory (if this is not the new IVF laboratory itself). The degree of isolation should be equivalent to an asbestos or lead abatement project. The isolation should be done through (i) physical barriers consisting of poly-sheeting supported by studding where needed; (ii) limited access to the construction area and the use of an access passageway with two doors in series; (iii) removal of all construction waste via an exterior opening or proper containment of waste before using an interior exit; (iv) negative air pressure in the construction area exhausting to the exterior, far removed from the laboratory's air intake and properly located with regard to the prevailing winds and exterior airflow; (v) extra interior fans during any painting or the use of adhesives to maximize removal of noxious fumes; and (vi) compiling and logging of a Safety Data Sheet (SDS; previously MSDS) for all paints, solvents, and adhesives in use.

Follow-up investigations with manufacturers and their representatives may be helpful because specifications of equipment may be changed without notice. The negative pressurization of the laboratory space requires continuous visual confirmation via a ball and tube pressure indicator or simply paper strips. Periodic sampling for particulates, aldehydes, and organics could be done outside the demolition and construction site, provided this is economically feasible. Alternatively, tracer gas studies can be done to verify containment. The general contractor of the demolition and construction should be briefed in detail on the need to protect the IVF facility and techniques to accomplish this. When possible, the actual members of the construction crew themselves should be selected and briefed in detail. Large filter units using filter

pellets of carbon and permanganate can be placed strategically. Uptake of organics can be assayed, but the frequency of routine filter changes should be increased during periods of construction activity.

Selection of building materials

Many materials release significant amounts of VOCs; a typical list includes paints, adhesives, glues, sealants, and caulking, which release alkanes, aromatics, alcohols, aldehydes, ketones, and other classes of organic materials. This section outlines steps to be taken to reduce these outgassing chemicals. Any and all interior painting throughout the facility should only be done on prepared surfaces with water-based paint formulated for low-VOC potential. During any painting, auxiliary ventilation should be provided using large industrial construction fans, with exhaust vented to the exterior. Paints that can significantly influence air quality should be emission tested (some suppliers already have these test results available). SDSs are generally available for construction materials. Suppliers should be encouraged to conduct product testing for emission potential. The variety of materials and applications complicates the testing process, but several procedures have been developed to identify and quantify the compounds released by building materials and furnishings. Interior paints must be water-based, low-volatile paints with acrylic, vinyl acrylic, alkyd, or acrylic latex polymers. Paints meeting this specification can also contain certain inorganic materials. Low-volatile paints may still contain low concentrations of certain organics. No interior paint should contain formaldehyde, acetaldehyde, isocyanates, reactive amines, phenols, and other water-soluble volatile organics. Adhesive glues, sealants, and caulking materials present some of the same problems as paints. None of these materials used in the interior should contain formaldehyde, benzaldehyde, phenol, and similar substances. Although water-based versions of these are generally not available, their composition varies widely. Silicone materials are preferred whenever possible, particularly for sealants and caulking work. A complete list of guidelines for material use during the construction of a tissue culture laboratory is available elsewhere [21].

“Burning in” of the finished facility

New IVF laboratories and new facilities around existing laboratories have often been plagued by complaints of occupants who experience discomfort from the chemicals released by new construction and furnishings. The ambient levels of many of these materials can be reduced by “burning in” the facility. A typical burn-in consists of increasing the temperature of the new area by 10°C–20°C and increasing the ventilation rate; even higher temperatures are acceptable. The combination of elevated temperature and higher air exchange aids in the removal of the volatile organics. Upon completion of the construction, the air handling system should be properly configured for the burn-in of the newly constructed area. As previously stated, the system must be capable of supplying the space with air at a temperature of 30°C–35°C, at less than 40% relative humidity. The burn-in period can range from 10 to 28 days, and the IVF laboratory should be kept closed during this time. If these temperatures cannot be reached by the base system, use auxiliary electrical heating to reach the minimum temperature. During burn-in, all lighting and some auxiliary equipment should be turned on and left running continuously. Naturally, ventilation is critical if

redistribution of irritants is to be avoided; the whole purpose is to purge the air repeatedly. Auxiliary equipment should of course be monitored during the burn-in.

The same burn-in principle applies to newly purchased incubators or other laboratory equipment. Removal of volatile organics is especially important in the critical micro-environment of the incubator. Whenever possible, it is advantageous to purchase incubators months in advance of their intended initial use and to operate them at an elevated temperature in a clean, protected location. An existing embryology laboratory is not a good space for the burn-in of a new incubator.

Most of the equipment available for use in an ART laboratory has not been designed or manufactured to be VOC-free. Special attention must be invested in new laboratory equipment to eliminate or reduce VOC levels by as much as possible before first use.

Most manufacturers do not address the issues of VOC outgassing in product manuals, even if the equipment has been expressly designed for the IVF field. Unpacking, cleaning, and operating equipment prior to final installation in a lab for outgassing the “new car smell” is always recommended.

Incubators should be unpacked, inspected, cleaned, outgassed, operated, recleaned, calibrated, and tested well in advance. The process can take several months to accomplish, but is generally a very essential task that is rewarded with the most suitable culture system that the selected incubator model can provide. When possible, operating incubators at elevated temperatures above the typical culture temperature will hasten the release or burn-off of VOCs. Extended operation at between 40°C and 45°C works well to burn off VOCs if this is within the manufacturer’s recommended temperature range. Incubator model VOC loads can vary greatly. Accurate VOC testing may be expensive and time-consuming, but it is recommended to test a specific incubator model to determine the new unit’s typical VOC characteristics and how much time outgassing may require.

Handheld VOC testing devices are available and can be used to help monitor the decline of total VOCs, but cannot match the level of accuracy of an environmental organic chemist’s testing. Handheld VOC meter technology generally is not sensitive enough to monitor low-molecular-weight classes of VOCs. They are reasonably affordable, easily used, and can provide a means of monitoring VOC reduction to help determine if the outgassing time may be sufficient to observe a reduction of VOCs.

New incubators are generally tested with a mouse embryo assay (MEA), replicating a culture system as part of a new incubator commissioning process. Most laboratories today use some variation of an oil culture system. The oil can serve as an excellent filter against potential VOCs but may not protect a culture system from the full range of VOC exposure, particularly low-molecular-weight compounds such as aldehydes. Incubator MEA commissioning should include both an oil and an open exposed media test to help evaluate the success of preparing the incubator. The dual MEA approach works well for humidified incubator systems, but may not be applicable if a dry, non-humidified culture system is used. Most dry, non-humidified culture systems are designed to recirculate chamber air and incorporate a VOC filtration strategy. Open culture generally cannot be used with non-humidified incubators. The manufacturer’s recommendations should be followed. Non-humidified incubators may require extended off-gassing and should be tested prior to use to confirm that they do not have a VOC issue. Chemical VOC filters should be replaced after burn-off prior to any MEA testing.

Laminar flow hoods and isolettes are also important potential VOC sources that should not be overlooked. They should be given ample time to operate and outgas as they can contribute to a lab’s VOC contamination load. High-efficiency particulate air (HEPA) and chemical filters should be selected for low-VOC manufacturing traits and also may require off-gassing. Care must be taken when outgassing laminar flow hoods and isolettes as they require a HEPA-filtered environment or replacement of their filters when transferred to an IVF lab.

After the burn-in is complete, commissioning of the IVF suite should be conducted to verify that the laboratory meets the design specifications. The ventilation and isolation of the laboratory should be verified by a series of tests using basic airflow measurements and tracer gas studies. The particulate levels should be determined to verify that the HEPA system is functional. Particulate sampling can be performed using US Federal Standard 209E. Microbial sampling for aerobic bacteria and fungi is often done in new facilities using an Andersen sampler followed by microbiological culturing and identification. The levels of VOC contamination should be determined. Possible methods are included in the US Environmental Protection Agency protocols using gas chromatography/mass spectroscopy and high-performance liquid chromatography that is sensitive at the microgram per cubic meter level [22–25].

Maintenance, planning, and sterilization

Even the best systems and designs will eventually fail unless they are carefully maintained. The heating, ventilation, and air conditioning (HVAC) will require filter changes, coil cleaning, replacement of drive belts, and chemical purification media. The most prevalent failure concerns the initial particulate filter. These are inexpensive filters designed to keep out large dust particles, plant debris, and insects, among other things. If such filters are not replaced promptly and regularly, they will fail, allowing the HVAC unit to become contaminated. The HEPA filters and chemical media also require inspection and periodic replacement. Maintenance staff should report their findings to the IVF laboratory.

The IVF laboratory must have a cleaning facility for surgical instruments. Ongoing use of an autoclave is not a problem as long as the released steam is rapidly exhausted to the outside. This keeps the relative humidity in the facility to controllable limits. Autoclaves should not be placed on the IVF laboratory’s HVAC system, but rather in a room that is built using tight construction and is exhausted directly outside of the building. The use of cold sterilizing agents is not advised. Aldehydes such as glutaraldehyde and *ortho*-phthalaldehyde from the autoclave can be transported inside the IVF laboratory.

Insurance issues

ARTs have become common practice worldwide and are regulated by a combination of legislation, regulations, or committee-generated practice standards. The rapid evolution and progress of ART reveal new legal issues that require consideration. Even the patient population is changing, as it becomes more acceptable for single persons and same sex/homosexual couples to seek and receive treatment. Donation of gametes, embryos, and gamete components; enforcement of age limits for treatment; selective fetal reduction; pre-implantation genetic diagnosis; surrogacy; and many other practices in ART present practitioners and

society at large with challenges, which are often defined by social norms, religion, and law and are specific to each country.

Furthermore, financial and emotional stresses often burden patients seeking treatment in countries where medicine is not socialized, and infertility treatment is not covered by insurance. This translates into an increasing number of ART lawsuits related to failed treatments in spite of generally improved success rates. Laboratory personnel and the laboratory owner should therefore obtain an insurance policy of a sufficiently high level and quality commencing prior to the first day of operations. Litigation-prone issues need special consideration, and include:

- Cancellation of a treatment cycle prior to egg retrieval
- Failure to become pregnant
- Patient identification errors
- Cryo-storage mishaps

These issues occur even if experienced practitioners consider themselves at low risk of exposure. Prior to engaging in the practice of ART, protocols must be established to identify potential problem areas and establish countermeasures.

Conclusions

It may be surprising how many professionals continue to pursue the establishment of new ART clinics at a time when competition is fierce, financial benefits are small, and existing ART services may appear to be approaching saturation in many areas and countries. Appearances can be misleading, however, and ART centres of excellence that deserve the trust and confidence of patients and serve as models for other practices are always needed.

This chapter provides some guidance for those who aspire to establish such outstanding, well thought out and planned ART practices. Although it cannot safeguard practitioners against adverse events, it introduces concepts in the proper design, construction, and operation of ART facilities that are of fundamental importance to treatment success; these guidelines have been painstakingly compiled through decades of practical experience and research. The approach is best adopted as a whole rather than dissected into its components and adopted in part or selectively. Keep in mind that resisting the urge to cut corners in the wrong places avoids future headaches and positions you and your patients on the path to success.

References

1. Jansen CA, van Beek JJ, Verhoeff A, Alberda AT, Zeilmaker GH. *In vitro* fertilisation and embryo transfer with transport of oocytes. *Lancet*. 1986;22:676.
2. Raffo FGE, Blaquier J. Transport IVF/ICSI: Results of a 25-year experience. *JBRA Assist Reprod*. 2018;22:123.
3. De Sutter P, Dozortsev D, Verhoeff A et al. Transport intracytoplasmic sperm injection (ICSI): A cost effective alternative. *J Assist Reprod Genet*. 1996;13:234–7.
4. Mortimer D, Cohen J, Mortimer SJ, Fawzy M, McCulloh DH, Morbeck DE, Pollet-Villard X, Mansour RT, Brison DR, Doshi A, Harper JC, Swain JE, Gilligan AV. Cairo Consensus on the IVF laboratory environment and air quality: Report of an expert meeting. *Reprod Biomed Online*. 2018;36:658–74. doi: [10.1016/j.rbmo.2018.02.005](https://doi.org/10.1016/j.rbmo.2018.02.005).
5. Kovačić B, Plas C, Woodward BJ, Verheyen G, Prados FJ, Hreinsson J, De los Santos MJ, Magli MC, Lundin K, Plancha CE. The educational and professional status of clinical embryology and clinical embryologists in Europe. *Hum Reprod*. 2015;30:1755–62.
6. Alikani M, Go KJ, McCaffrey C, McCulloh DH. Comprehensive evaluation of contemporary assisted reproduction technology laboratory operations to determine staffing levels that promote patient safety and quality care. *Fertil Steril*. 2014;102:1350–6.
7. Abdalla HI, Bhattacharya S, Khalaf Y. Is meaningful reporting of national IVF outcome data possible? *Hum Reprod*. 2010;25:9–13.
8. Intra G, Alteri A, Corti L, Rabellotti E, Papaleo E, Restelli L, Biondo S, Garancini MP, Candiani M, Viganò P. Application of failure mode and effect analysis in an assisted reproduction technology laboratory. *Reprod Biomed Online*. 2016;33:132–9.
9. Cimadomo D, Ubaldi FM, Capalbo A, Maggiulli R, Scarica C, Romano S, Poggiana C, Zuccarello D, Giancani A, Vaiarelli A, Rienzi L. Failure mode and effects analysis of witnessing protocols for ensuring traceability during PGD/PGS cycles. *Reprod Biomed Online*. 2016;33:360–9.
10. Cohen J, Gilligan A, Esposito W, Schimmel T, Dale B. Ambient air and its potential effects on conception *in vitro*. *Hum Reprod*. 1997;12:1742–9.
11. Cohen J, Gilligan A, Willadsen S. Culture and quality control of embryos. *Hum Reprod*. 1998;13(Suppl 3):137–44.
12. Fox JT, Ni P, Urrutia AR, Huynh HT, Worrilow KC. Modelling the equilibrium partitioning of low concentrations of airborne volatile organic compounds in human IVF laboratories. *Reprod Biomed Online*. 2023;46:54.
13. Heitmann RJ, Hill MJ, James AN, Schimmel T, Segars JH, Csokmay JM, Cohen J, Payson MD. Live births achieved via IVF are increased by improvements in air quality and laboratory environment. *Reprod Biomed Online*. 2015;31:364–71.
14. Esteves SC, Bento FC. Implementation of cleanroom technology in reproductive laboratories: The question is not why but how. *Reprod Biomed Online*. 2016;32:9–11.
15. Swain JE. Decisions for the IVF laboratory: Comparative analysis of embryo culture incubators. *Reprod Biomed Online*. 2016;28:535–47.
16. Alikani M, Cohen J, Tomkin G et al. Human embryo fragmentation *in vitro* and its implications for pregnancy and implantation. *Fertil Steril*. 1999;7:836–42.
17. Navarro PA, Liu L, Trimarchi JR et al. Noninvasive imaging of spindle dynamics during mammalian oocyte activation. *Fertil Steril*. 2005;83(Suppl 1):1197–205.
18. Hall J, Gilligan A, Schimmel T, Cecchi M, Cohen J. The origin, effects and control of air pollution in laboratories used for human embryo culture. *Hum Reprod*. 1998;13(Suppl 4):146–55.
19. Boone WR, Johnson JE, Locke AJ, Crane MM 4th, Price TM. Control of air quality in an assisted reproductive technology laboratory. *Fertil Steril*. 1999;71:150–4.
20. Morbeck DE. Air quality in the assisted reproduction laboratory: A mini-review. *J Assist Reprod Genet*. 2015;32:1019–24.
21. Gilligan A. Guidelines for Material Use in the USA during Construction of a Tissue Culture Laboratory. Emerson, NJ: Alpha Environmental, 2015.
22. Seifert B. Regulating indoor air. Presented at the 5th International Conference on Indoor Air Quality and Climate, Toronto, Canada, 1990; 5:35–49.
23. Sarigiannis DA, Karakitsios SP, Gotti A et al. Exposure to major volatile organic compounds and carbonyls in European indoor environments and associated health risk. *Environ Int*. 2011;37:743–65.
24. Federal Standard 209E. Washington, DC: General Services Administration, US Federal Government, 1992.
25. Compendium of Methods for the Determination of Toxic Organic Compounds in Ambient Air, US EPA 600/4-84-041, April 1984/1988. [Available from the US EPA through the Superintendent of Government Documents, Washington, DC.]

2

QUALITY CONTROL

Maintaining Stability in the Laboratory

Ronny Janssens, Neelke De Munck, and Johan Guns

Introduction

It has now been almost 40 years since *in vitro* fertilization (IVF) was developed by Edwards and Steptoe. Over these decades, practice in medically assisted reproduction (MAR) has evolved from a new, experimental procedure into a well-established routine treatment of infertility driven by the development of new procedures such as intracytoplasmic sperm injection (ICSI), extended culture, pre-implantation genetic testing (PGT), vitrification, ongoing research, the development of better and safer products and culture media, more stringent quality control programs by commercial companies, and a better understanding of possible factors that might have an impact on the outcome of the procedure. Although success rates have improved over time, it is hard to define which laboratory practices contribute to this success [1]. In a survey of US high-performing centers, factors that were identified as being vital to excellent outcomes were experience of physicians, embryologists, and staff members, along with consistency of approach, attention to detail, and good communication.

Together with the evolution from research toward worldwide routine application, we have seen increasing regulatory requirements and the development of professional standards for embryology laboratories. In the beginning of this century, both US and European authorities issued regulations to ensure the quality and safety of human tissues and cells, and now the European Union Tissues & Cells Directive 2004/23/EC (EUTCD) [2] has been implemented in all EU member states.

Although the legislation differs between the US and Europe and the interpretation and translation into national legislation of the EUTCD in the EU member states is different from country to country, there is a common requirement to implement a quality management system (QMS) in any ART laboratory.

All is well, until disaster strikes you. Remember Captain Smith, a very experienced captain on the helm of the Titanic when it sunk in 1912. Sometimes things do not go as expected and disasters or errors occur. All embryologists are or will be confronted with Murphy's Law: if anything can go wrong, it will go wrong. It is our challenge and professional duty to beat Murphy's Law and be better than Captain Smith and here, quality management can help.

Although sometimes seen as a burden, quality management supports a successful clinic. It is a tool to avoid unwanted and uncontrolled fluctuations in a process and ensures the consistency of approach and attention to detail so that stable results can be achieved over time. Essential elements of quality management (and relevant standards for quality management) leading to standardization are risk management, validation, standard operating procedures, communication, and training.

Risk management

Treatment is influenced by internal and external factors that create uncertainty in achieving the desired outcome. The effect of

this uncertainty is "risk." Prospective risk management [3] is an instrument dealing with the possibility that some future event(s) might cause harm [4]. It includes strategies and techniques for recognizing and confronting any such threat and provides a disciplined environment for proactive decision-making (or beating Murphy's Law). Risk management is now an essential element of accreditation or certification standards and is even mandatory for some regulatory authorities such as the UK Human Fertilisation and Embryology Authority (HFEA) [5] and European Directorate for the Quality of Medicines & Healthcare (EDQM) [6]. In a risk assessment procedure, you identify what the risks are, what would be the cause, what would be the consequence, and what controls could be in place to minimize risk. There are many risk assessment techniques [7], but the two most commonly used are "failure mode and effects analysis (proactive)" and "fault tree analysis (retrospective)." It is good practice to perform a proactive risk assessment before introducing or changing a procedure. The Euro Good Tissue & Cell Practices (Euro GTP II) provides practical tools to evaluate and quantify risks [8]. Once risks are identified, they can be controlled or treated so that the likelihood or the consequence (impact) of an event is reduced. A good example of proper risk treatment in the IVF laboratory is the installation of a real-time equipment monitoring system (EMS). The EMS increases the detection of equipment malfunctioning and reduces the consequence by warning in time so that loss of valuable biological material can be prevented. Although there is an important investment cost to installing a real-time EMS, it has been demonstrated that, even for a small laboratory, an automated system can represent not just increased functionality, but it also saves money within three years [9]. Monitoring and alarming are essential tools for quality control and maintaining stability in the laboratory and are also required by EU directive 2006/86/EC [10], ISO 15189:2012 [11], and the HFEA code of practice [5].

Validation

The ISO definition of validation is "confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled." IVF is a process (a set of interrelated or interacting activities that transform inputs into outputs). A basic objective of validation is to ensure that each step and each variable of the process is identified and controlled and process variability is reduced so that the finished product meets customer requirements (e.g., consistent high pregnancy rates).

The US Food and Drug Administration (FDA) [12] and EDQM [6] published guidelines that outline the general principles for process validation. Quality, risks, safety, and efficacy should be considered from the design phase of a process. Certainly in IVF, the quality of the "end product" cannot be measured, so each contributing factor (infrastructure, equipment, and utilities) and all the steps of the process need to be known and controlled.

Ideally, prospective validation is preferred, but certainly in existing IVF clinics this is not always possible. According to EDQM, establishments performing very simple, minimal manipulation of a limited range of tissues and cells in accordance with published methods, or following long-established practices using the same materials and equipment, may rely on ongoing quality control and periodic reviews. Such establishments should still document their validation policy, explaining their approach on the basis of risk assessment, and should perform a retrospective verification of their critical processes to confirm that the method has the intended (clinical) outcome. The qualification of premises, equipment, suppliers, software, materials, consumables, reagents, and personnel should be ensured and should result in written reports. During the validation, in-process controls should be defined in order to monitor the process.

Process validation is needed before the introduction (process design) of a new method into routine use, whenever the conditions change for which a specific method has been validated (other instruments, changes in environment, etc.), and whenever the method is changed [12, 13].

During routine use, periodic verification of critical parameters and, when technically feasible by using modern technology, continuous process verification are necessary to ensure that the process remains in a state of control. Examples of laboratory processes that need to be validated are cleaning and decontamination procedures, sperm processing, IVF/ICSI, egg collection, embryo culture, cryopreservation, and embryo replacement.

In addition, equipment needs to be qualified in order to provide a high degree of assurance that it will consistently meet its predetermined specifications with minimal variation. Equipment qualification is broken down into three phases: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). IQ is the first step and ensures that the equipment is correctly installed according to the manufacturer's specifications. As an example, a new incubator needs to be installed on a solid, vibration-free surface, the room temperature should be within a defined range, and the instrument should be connected to CO₂ and the main power. During the next step, OQ, the equipment is calibrated and tests are performed in order to document a baseline of the critical parameters of the equipment. For an incubator, this is defining set points for CO₂, temperature, and oxygen and a verification of these parameters with independent, calibrated measuring equipment. The PQ phase then tests the ability of the incubator to perform over long periods within an acceptable tolerance range. The equipment, utility, and system should then be maintained, monitored, and calibrated according to a regular schedule by responsible personnel with appropriate qualifications and training. Parameters of calibration and equipment qualification should be traceable to international standards. Calibrated equipment should be labeled, coded, or identified so that the calibration status and recalibration due date are clear. If equipment is not used for a certain period of time, then the calibration status needs to be verified before use.

Documentation

Good documentation is an essential part of any QMS. The process validation and equipment qualification and laboratory standard operating procedures need to be correctly and completely documented. These documents should be approved by the laboratory director or a delegated manager, regularly reviewed, and updated. Before any new or changed procedure may be introduced into

routine, staff should be trained; the training should be specific and focused on the role of the employee.

Change control

The core principle of quality management is about change; change for continuous improvement or the plan, do, check, act cycle. Whenever processes or procedures are changed, the impact of the change should be justified and documented in order to prove that the change does not adversely affect the process.

Change control is a systematic approach that is used to ensure that any intended modification to the process, equipment, instruments, facility, and so on, is introduced in a coordinated manner and to reduce the possibility that unwanted or unnecessary changes will be introduced into the culture system [14].

Unplanned deviation from these approved processes or documents with potential impacts on quality, safety, or efficacy should be registered as non-conformity in the QMS.

The key principle of change control is to understand and document what was done, why, when, where, by whom, how, and what were the results.

Changes potentially requiring revalidation or clinical testing prior to implementation (decision based on risk analysis) are changes of facilities and installations, which may influence the process (cleanrooms or heating, ventilation, and air conditioning [HVAC]), changes in materials (puncture needles or transfer catheters) or reagents (culture media), changes in the process itself (implementation of new technology or findings based on current knowledge), changes in equipment, or support system changes (cleaning, supply, or information technology). All changes that have the potential to impact quality, safety, and efficacy should be justified, documented, approved (or rejected), communicated, and made known to laboratory staff and implemented in practice.

A change request procedure should be incorporated into the QMS.

Quality control and quality assurance

There is only one thing that is truly important in an IVF laboratory: everything. This was the conclusion of an international expert meeting to establish consensus guidelines on IVF culture conditions [15]. In this report, more than 50 consensus guideline points were established on different topics: embryo culture—basic principles and interactions; temperature in the IVF laboratory; humidity in culture; carbon dioxide control and medium pH; oxygen tension for embryo culture; workstations—design and engineering; incubators—maintaining the culture environment; micromanipulation—maintaining a steady physico-chemical environment; handling practices; assessment practices; culture media—buffering and pH, general composition and protein supplementation, sequential or single step media for human embryo culture; use and management—cold chain and storage; test equipment—calibration and certification; and laboratory equipment and real-time monitoring.

Having established in detail for your protocols which aspects of the process are important to delivering the required quality (by proper validation), it is necessary that in-process controls are properly monitored. Regular monitoring of key performance indicators [16] provides good evidence of a clinic's performance, but, unfortunately, a real decline in pregnancy rates may only be detected very late. It is therefore crucial to establish strict quality control procedures, routines, and controls to ensure that

procedures and pieces of equipment operate appropriately and the process remains “in control.”

This section focuses on the instruments and techniques used to document environmental and process parameters such as temperature and gas concentrations, and discusses the quality control and quality assurance of laboratory personnel, infrastructure, equipment, culture media, and contact materials.

Infrastructure and environment

Cleanrooms and air quality

The relation between environmental toxicants and fertilization and embryo development has been reported by several authors [17, 18]. More recently, positive pressure in the lab, high-efficiency particulate air (HEPA) filtration of laboratory air, filtration for volatile organic compounds (VOCs), and use of chemical active compounds are identified as factors that are common in high-performing IVF programs [1]. Most modern IVF centers are now located in cleanrooms.

HVAC functioning of a cleanroom should be monitored by a building monitoring system (BMS). The air quality requirement can vary from country to country, but most modern IVF laboratories are housed in cleanrooms classified ISO 8 to ISO 7 or EU GMP D to C, although there is now international consensus to aim for GMP C for ART laboratories [19]. Once the clinic has specified its air quality requirements, compliance with the designated classification has to be demonstrated in the formal process of qualification. Qualification is mostly done on a yearly basis by a testing organization that performs normative tests compliant with the ISO 14644-1 and ISO 14644-3 standards [20]. Qualification is followed by monitoring in order to control performance, both in rest state and in operation [21].

Air quality monitoring [22] consists of the enumeration of particles and microorganisms, both in rest and in activity. Before starting, a monitoring program needs to establish the sampling frequency and locations, the number of samples per location, the sample volume, and the test methods. This way of working, which is not yet familiar to ART and other tissue/cell establishments, derives from pharmaceutical guidelines. The EU directive refers to annex 1 of the EU GMP [23] that specifies the techniques for particle and microbial testing, similar to those described in the US cGMP [22] and the US Pharmacopeia for the production of medicines for human use [24]. These pharmaceutical guidelines can guide the ART establishments in setting up a monitoring program.

Furthermore, the EU GMP makes a distinction between environmental monitoring at rest state and monitoring of the aseptic process in operation. Environmental monitoring at rest state verifies whether the environment is ready for the forthcoming activity, whereas aseptic process monitoring aims to ensure that the people, processes, and environment remain under control during operation.

Particle counters can be part of a BMS or an EMS. It is possible to monitor VOCs in laboratory air. Photo ion VOC detectors, measuring in the ppm range and with a 4- to 20-mV output, are commercially available and can easily be connected to any real-time EMS. Monitoring VOCs may lead to the detection of non-compliance of cleaning and disinfection procedures by cleaning staff outside of working hours and can avoid the introduction of dangerous and toxic products released by non-approved cleaning agents.

The maintenance schedule for serving and filter replacement should be defined (by particle count for HEPA filters and analysis of filter saturation for active carbon and chemical VOC filters) and records should be kept of filter replacement dates and batch numbers. The preventive maintenance schedule should be

defined in a service-level agreement between the laboratory and the company performing the maintenance.

Temperature and relative humidity

The absolute value of ambient temperature in the cleanroom is not really important for MAR (occupational health and safety rules should be respected) but should not exceed 25°C in order to control microbial contamination. If the environment of a cleanroom is cold and dry, microbiological contaminants will not grow. If the ambient relative humidity (RH) and temperature of the cleanroom environment exceeds 50% and 25°C, the risk of bacteria growth increases. On the other hand, humidity that is below 35% promotes static electricity, personal discomfort, and irritation of mucous membranes and eyes. An ambient RH between 40% and 50% minimizes the impact of bacteria and respiratory infections and provides a comfortable working environment. Also, incubators do not function well if ambient temperature is above 30°C. However, for optimal lab performance, it is important to keep ambient temperature constant to avoid fluctuations in the surface temperature of equipment (heated stages and incubators), and therefore the ambient temperature should be monitored and alarmed.

During the design phase of a new cleanroom, attention should be given to the positioning of workstations and incubators so that they are not located directly in front of or below HEPA filtered air conditioning outlets.

Ambient temperature and RH are usually monitored by a BMS.

Light

The effect of direct sunlight and hard white fluorescent light on mammalian zygotes and embryos is well documented [25, 26] and most laboratories limit the amount of light exposure to gametes and embryos. In total, 95% of this light energy originates from microscope halogen lamps during manipulation and handling [27], and, in particular, the blue region (400–500 nm) of light is harmful [28]. Therefore, the use of green filters on microscopes is recommended.

Gas supplies

There is now convincing evidence that low oxygen concentrations for embryo culture are associated with increased live birth rates [29]. IVF incubators depend on a supply of gas in order to regulate their internal atmospheres. Depending on the incubator's design, this is either 100% CO₂ and 100% N₂ for incubators with integrated gas mixing units, or custom-made mixtures of 5%–6% CO₂, 5% O₂, and 89%–90% N₂ for incubators without integrated gas mixing capacity (MINC™ benchtop incubator, Cook Medical; BT37 benchtop incubator, Origio/Planer). All gases should be of the highest quality and VOC filters should be installed on gas lines. Incubators with gas mixing units do have sensors and can give an alarm when the gas supply is failing, but this is not the case with incubators that run on premixed gasses. The latter can be monitored by placing a small Petri dish-sized infrared CO₂ sensor [30] inside an incubator chamber.

Laboratory equipment and real-time monitoring

Real-time monitoring (RTM) has long been seen as simply impractical because of the lack of accurate CO₂ sensors, the difficulty in connecting too many points, and the cost of cabling and adding sensors and data transmitters. Today, with the universal availability of low-cost wireless technology, the internet, smartphones, and tablets, this is no longer the case, and there are now affordable solutions that provide vital, real-time information to

monitoring systems and the people who need it, such as the laboratory manager. RTM systems can reduce “loss” by equipment failure and thus provide the manager with increased safety and reliability. Also, regulators see the benefits of monitoring; this requirement is now integrated into professional guidelines [31], regulatory requirements [10], and accreditation standards [11].

It is possible to connect analogue sensors for temperature and gas levels (CO_2 , O_2 , and VOCs). If feasible, monitoring sensors that are independent from the equipment should be used. This makes it possible to detect equipment sensor drift, allows verification of manufacturers’ performance claims, and may detect environmental factors such as electrical failure.

Air pressure, RH, airflow sensors, and particle counters can be connected to an RMS, but these parameters are usually integrated into a BMS. Laboratory ambient air monitoring should be part of the EMS since deviations in ambient temperate have consequences on the temperature regulation of microscope heated stages.

Digital signals that can be monitored in real time include door status and equipment alarm signals. It is even possible to read digital Recommended Standard (RS) 232 or RS 485 interphases.

Modern web-based systems provide accurate and effective control of equipment. The data are remotely accessible over a secure internet connection and intelligent alarms warn the laboratory manager in case of an unexpected event or equipment malfunctioning or failure. To increase reliability, technical alarms (sensor break, monitoring equipment failure, or network failure) should be possible, and this aspect should be considered when a monitoring system is chosen. Of course, with modern technology, it is possible to send alarms by telephone, email, or SMS, but the alarm messaging program should be bi-directional so that alarm acknowledgement is possible (and logged). In case of no reaction within a predefined timeframe, an automatic cascading system should be activated.

Culture system

Temperature issues

Although the optimal temperature for oocyte handling and embryo culture is not really known, limited decreases in temperature can alter the cytoskeleton [32] and spindle [33] of oocytes, and there is limited recovery after cooling and rewarming [34], indicating that human meiotic spindles are exquisitely sensitive to alterations in temperature and that the maintenance of temperature close to 37°C during *in vitro* manipulations is important for normal fertilization and subsequent embryo development. These temperature effects are irreversible so it is important to avoid suboptimal temperatures. Temperature issues can occur

during follicle puncture, during manipulations on heated stages on stereo microscopes and injection microscopes, in incubators, and during embryo transfer. Temperature should be measured in culture dishes under oil and in tubes with calibrated probes. The choice of measuring probe is important. Thin, fast-responding, non-shielded type T thermocouples can be used to detect small temperature gradients and are excellent at detecting hotspots on heating stages, whereas more precise, small probes fixed in culture dishes are more suitable for precise temperature measurements in incubators. Although the most stable and accurate sensors are resistance temperature detectors (Pt100 and Pt1000), they are not easily available in small sizes to affix inside a culture dish. For this purpose, thermistor probes are probably a better choice. Thermistors with 0.1°C accuracy are now widely available and at a very reasonable price. They have a fast response time and because of their high sensitivity, they are ideal in detecting temperature changes in culture dishes. Of course, accurate temperature measurement is only possible through the use of suitably calibrated sensors and instruments, and the accuracy of these measurements will be meaningless unless the equipment and sensors are correctly used. Good knowledge of measurement science is a basic requirement, one that is lacking in many laboratories.

Culture media and pH

The choice of culture medium is beyond the scope of this chapter. There is no ideal pH for culture media, as this varies from medium to medium and manufacturer to manufacturer, but it usually fluctuates within a range of 7.1 to 7.4. The pH of bicarbonate-buffered medium is regulated by the concentration of CO_2 dissolved in the culture medium, and this is regulated by the partial pressure of CO_2 in the incubator air. It is therefore important to carefully monitor incubator performance by RTM. In large-volume standard incubators, it is easy to integrate infrared CO_2 sensors. In small-volume desktop incubators, this is more challenging, but in some brands it is possible. While with modern and well-controlled incubators it is possible to maintain stable pH values, pH will increase while culture media are outside the incubator. To slow down this pH increase, oil is often layered over culture media. Besides this protective effect on pH, an oil overlay also reduces evaporation and heat loss and provides protection from particulate air contaminants [35].

The protective effect on pH is, however, quite limited in time, as shown in Figure 2.1. When culture medium is directly exposed to ambient air, the pH rise starts immediately. When a culture dish is removed from the incubator and the lid is left on the dish,

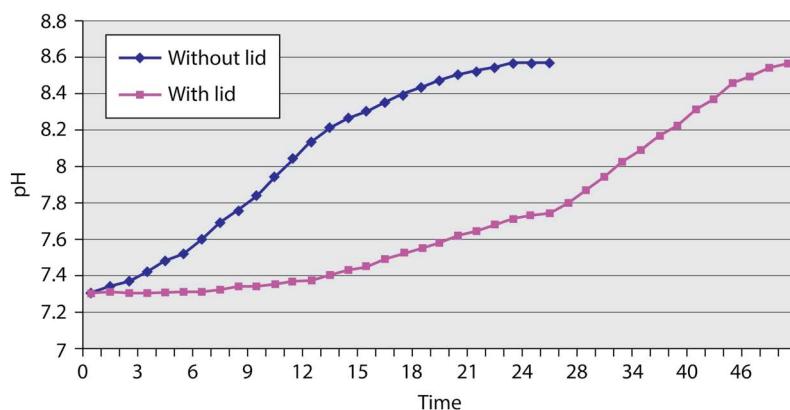


FIGURE 2.1 pH of culture medium under oil in ambient air over time (minutes).

then the pH starts to rise after 10 minutes. It is therefore good practice to leave lids on culture dishes during zygote and embryo scoring. These pH problems can be avoided by working in isolators with CO₂ (and temperature) regulation.

For quality control purposes, the pH of each batch of culture medium should be measured after proper pre-equilibration and should be within the range specified on the certificate of analysis. A conventional pH meter with a glass electrode is technically challenging (samples for measurement have to be removed from the incubator, measurements should be done at 37°C, and measurements have to be done in ambient air) and time-consuming. Better solutions are the continuous pH recorders in incubators or the use of a point-of-care blood gas analyser (only possible for culture media, not suitable for HEPES or MOPS buffered media).

Osmolality

The osmolality of commercial media ranges from 255 to 298 mOsm/kg [36]. Most IVF labs culture embryos in droplets of media under oil overlay. Microdrop preparation can influence culture media osmolality, which can impair embryo development [37], so this technique should be standardized and staff should be trained in culture dish preparation. With recent developments such as non-humidified benchtop incubators and single-step media with prolonged culture without medium change, monitoring osmolality has become an important part of quality control (after opening and during storage) and process control (measurement of spent culture medium and after five to six days of culture). For this purpose, each laboratory should have a freezing point depression osmometer.

Contact materials

Disposables such as oocyte retrieval needles, culture dishes, ICSI needles, and transfer catheters are used extensively throughout the whole IVF procedure, and the choice of disposable should be defined and its performance documented in a validation procedure. Disposables for embryo culture are available from many different manufacturers. Unfortunately, some have been shown to have toxic effects on gametes and embryos by sperm motility assay [38] or mouse embryo assay (MEA) [39].

The American Society for Reproductive Medicine (ASRM) practice guidelines [40] require that material that comes in contact with sperm, eggs, or embryos should be non-toxic and should be tested by the vendor with an appropriate bioassay or animal model. In Europe, all materials and reagents that come into contact with human material for transplantation must be approved as a medical device (MDR) [41]. This includes, but is not limited to, aspiration needles, transfer catheters, plastic ware, glassware, culture media, and protein sources. The European Society of Human Reproduction and Embryology (ESHRE) guidelines [31] require that culture media should be mouse embryo tested, and the European Directives require that these disposables should be tested with an adequate bioassay by the supplier, but there is no consensus or standard on how this MEA test should be performed. Variables that have an effect on MEA sensitivity [42] are the starting point (oocytes, zygotes, or two-cell embryos), number of embryos per volume of culture medium, culture medium and use of albumin, exposure protocol of the disposable (medium volume and duration of exposure), and the use of an oil overlay, so manufacturers can easily modify their assay conditions and either aim to maximize sensitivity (with a high rejection rate) or reduce sensitivity (with a low rejection rate). Product inserts or certificates of analysis are not informative and the end-user cannot

judge the real value of the company's statement "MEA tested." Laboratories should therefore request this information and select suppliers based on their transparency in providing information on test conditions, exposure protocols, and acceptance criteria.

Laboratory personnel

The number and qualifications of laboratory personnel are critical factors for maintaining stability in the laboratory. The recommended staffing levels are one full-time equivalent "bench" or "hands-on" embryologist per 120 stimulation cycles per year [43]. As in any discipline in which technical proficiency can directly influence a measurable outcome, monitoring performance is essential to confirm that a procedure is carried out correctly and optimally. The aim of this monitoring is to discover departures from protocol and to identify opportunities for correction and improvement. Examples of performance parameters are the number of two pronuclei and the number of degenerated oocytes per total number of mature eggs injected (ICSI), number of embryos recovered intact and viable per number cryopreserved and per number thawed/warmed (cryopreservation or vitrification), number of clinical pregnancies per number of embryo transfers (embryo transfer), number of embryos continuing development per number of embryos biopsied, number of embryos with molecular signals per number of embryos biopsied (embryo biopsy), number of oocytes survived and intact per number of oocytes vitrified (oocyte vitrification), and number of gestational sacs per total number of hatched embryos [44].

Witnessing

One of the definitions of quality is to satisfy stated or implied needs or, in other words, to meet patients' expectations. Traceability of cells during IVF is a fundamental aspect of treatment, and involves witnessing protocols. Failure mode effect analysis of a human double-witness system has clearly demonstrated the loopholes and risks of manual witnessing [45]. Automated electronic systems based on barcodes or radiofrequency identification tags can replace manual witnessing [46] and reduce the risk of gamete exchange. It is our experience that such an electronic witnessing system reduces staff distraction and stress, increasing staff efficiency.

References

1. Van Voorhis B, Thomas M, Surrey E, Sparks A. What do consistently high-performing *in vitro* fertilization programs in the U.S. do? *Fertil Steril*. 2010;94:1346–9.
2. Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. Official Journal of the European Union 7.4.2004.
3. ISO 31000:2009. Risk management—Principles and guidelines, 2009.
4. Mortimer D, Mortimer ST. Quality and Risk Management in the IVF Laboratory. Cambridge: Cambridge University Press, 2005.
5. Human Fertilisation and Embryology Authority. *Code of Practice*, 8th edition, 2015. www.cambridge.org
6. <https://www.edqm.eu>. Accessed on 17/02/2021.
7. ISO 31010:2009. Risk management—Risk assessment techniques. 2009.
8. <http://www.goodtissuepractices.eu/>. Accessed on 17/02/2022.
9. Mortimer D, Di Bernardino T. To alarm or monitor? A cost-benefit analysis comparing laboratory dial-out alarms and a real-time monitoring system. Alpha Newsletter. 2008;August:1–7.

10. Directive 2006/86/EC implementing Directive 2004/23/EC of the European Parliament and of the Council as regards traceability requirements, notification of serious adverse reactions and events and certain technical requirements for the coding, processing, preservation, storage and distribution of human tissues and cells. Official Journal of the European Union 25.10.2006.
11. ISO 15189:2012. Medical laboratories—Requirements for quality and competence, 2012.
12. FDA (CDER, CBER, CVM). Process Validation: General Principles and Practices, 2011.
13. Ludwig H. Validation of Analytical Methods and Procedures. <http://www.labcompliance.com/tutorial/methods/>
14. OECD Principles of Good Laboratory Practices. <http://www.oecd.org/chemicalsafety/testing/oecdseriesonprinciplesofgoodlaboratorypracticeglpandcompliancemonitoring.htm>
15. Cairo 2018 Consensus Group, "There is only one thing that is truly important in an IVF lab: Everything" Cairo consensus guidelines on IVF culture conditions. Reprod BioMed Online. 2020.
16. ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine. Electronic address: Coticchio.biogenesisi@grupposandonato.it. The Vienna consensus: Report of an expert meeting on the development of ART laboratory performance indicators. Reprod Biomed Online. 2017;35(5):494–510. doi: [10.1016/j.rbmo.2017.06.015](https://doi.org/10.1016/j.rbmo.2017.06.015).
17. Cohen J, Gilligan A, Esposito W, Schimmel T, Dale B. Ambient air and its potential effects on conception. Hum Reprod. 1997; 12:742–9.
18. Johnson JE, Boone WR, Bernard RS. The effects of volatile compounds (VC) on the outcome of *in vitro* mouse embryo culture. Fertil Steril. 1993;Suppl 1:S98–9.
19. Mortimer D, Cohen J, Mortimer S, Fawzy M, McCulloch D, Morbeck D, Pollet-Villard X, Mansour R, Brison D, Doshi A, Harper J, Swain J, Gilligan A. Cairo Consensus on the IVF laboratory environment and air quality: Report of an expert meeting. Reprod BioMed Online. 2015;4:516–22.
20. International Standard Organisation. Cleanrooms and associated controlled environments—Part 1: *Classification of air cleanliness by particle concentration*, ISO 14644-1, 2015.
21. International Standard Organisation. Cleanrooms and associated environments—Part 2: *Monitoring to provide evidence of cleanroom performance related to air cleanliness by particle concentration*, ISO 14644-2, 2015.
22. Guns J, Janssens R, Vercammen M. Air quality management. In: Practical Manual of In Vitro Fertilization: Advanced Methods and Novel Devices. Nagy ZP, Varghese AC, Ashok A (eds.). New York, NY: Springer, pp. 17–25, 2012.
23. European Union. EU Good Manufacturing Practice. Medicinal Products for Human and Veterinary Use. Annex 1: Manufacture of Sterile Medicinal Products (corrected version), 91/356/EEC, 2008.
24. USP29-NF24. Microbiological Evaluation of Clean Rooms and Other Controlled Environments. Baltimore, MA: United States Pharmacopeia, 2010.
25. Schumacher A, Fischer B. Influence of visible light and room temperature on cell proliferation in preimplantation rabbit embryos. J Reprod Fertil. 1988;84:197–204.
26. Takahashi M, Saka N, Takahashi H, Kanai Y, Schultz RM, Okano A. Assessment of DNA damage in individual hamster embryos by comet assay. Mol Reprod Dev. 1999;54:1–7.
27. Ottosen LDM, Hindkjar J, Ingerslev J. Light exposure of the ovum and preimplantation embryo during ART procedures. J Assist Reprod Genet. 2007;24:99–103.
28. Korhonen K, Sjövall S, Viitanen J, Ketoja E, Makarevich A, Pēippo J. Viability of bovine embryos following exposure to the green filtered or wider bandwidth light during *in vitro* embryo production. Hum Reprod. 2009;24:308–14.
29. Bontekoe S, Mantikou E, vanWely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. Cochrane Database Syst Rev. 2012;7:CD008950.
30. <https://planer.com/products/petrisense-ph-co2-sensor.html>
31. Magli MC, Van den Abbeel E, Lundin K, Royere D, Van der Elst J, Gianaroli L. Revised guidelines for good practice in IVF laboratories. Hum Reprod. 2008;23:1253–62.
32. Almeida PA, Bolton VY. The effect of temperature fluctuations on the cytoskeletal organization and chromosome constitution of the human oocyte. Zygote. 1995;3:357–65.
33. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. Fertil Steril. 1990;54:102–8.
34. Wang WH, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. Hum Reprod. 2001;16:2374–8.
35. Characterization and comparison of commercial oils used for human embryo culture. Hum Reprod. 2022 Jan 28;37(2):212–25.
36. Swain JE, Pool TB. Culture media in IVF: Decisions for the laboratory. In: Practical Manual of In Vitro Fertilization: Advanced Methods and Novel Devices. Zsolt PN, Alex CV, Ashok A (eds.). New York, NY: Springer, 2012.
37. Swain JE, Cabrera L, Xu X, Smith GD. Microdrop preparation factors influence culture-media osmolality, which can impair mouse embryo preimplantation development. Reprod Biomed Online. 2012;24:142–7.
38. Nijs M, Franssen K, Cox A, Wissmann D, Ruis H, Ombelet W. Reprotoxicity of intrauterine insemination and *in vitro* fertilization-embryo transfer disposables and products: A 4-year survey. Fertil Steril. 2009;92:527–35.
39. Van den Abbeel E, Vitrier S, Lebrun F, Van Steirteghem A. Optilized bioassay for the detection of embryology contaminants. Hum Reprod. 1999; 114.
40. The Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology. Revised guidelines for human embryology and andrology laboratories. Fertil Steril. 2008;90(Suppl 3): S45–S59.
41. Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on medical devices, amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC. Official Journal of the European Union 5.05.2017.
42. Boone WR, Higdon HL, Johnson JE. Quality management issues in the assisted reproduction laboratory. J Reprod Stem Cell Biotechnol. 2010;1:30–107.
43. Alpha Scientists in Reproductive Medicine. The alpha consensus meeting on the professional status of the clinical embryologist: Proceedings of an expert meeting. Reprod Biomed Online. 2015;30:451–61.
44. Go KJ. 'By the work, one knows the workman': The practice and profession of the embryologist and its translation to quality in the embryology laboratory. Reprod Biomed Online. 2015;31:449–58.
45. Rienzi L, Bariani F, Dalla Zorza M, Romano S, Scarica C, Maggiulli R, Nanni Costa A, Ubaldi FM. Failure mode and effects analysis of witnessing protocols for ensuring traceability during IVF. Reprod Biomed Online. 2015;31:516–22.
46. Thornhill AR, Brunetti XO, Bird S, Bennett K, Rios LM, Taylor J. Reducing human error in IVF with electronic witnessing. Fertil Steril. 2011;96(3):S179.

3

KPIs FOR THE IVF LABORATORY

Alison Campbell

Introduction

Key performance indicators (KPIs) are essential tools to monitor and control quality and improvements in the in vitro fertilization (IVF) laboratory.

Numerous variables play into the success and demonstrable performance of an IVF laboratory. These variables include patient management, gamete quality, laboratory and culture environment, equipment, processes, consumables, and the skill and degree of experience of the laboratory team. The IVF laboratory plays a key role in the success of IVF treatments and therefore is often the focus of great scrutiny. In addition, a high number of outcome measures can be used to measure the success of a laboratory, making the design and selection of KPIs complex.

The simplest and arguably the most practical and effective approach to KPI use is to identify high-level, or headline, KPIs, with defined minimum numbers of processes or treatments to enable reliable analysis. Alongside these, the facility to drill down deeper and further into additional indicators, if required, in order to pinpoint and address a potential weakness in the system is beneficial.

These headline KPIs can be useful to identify areas or processes which may need further scrutiny, or to facilitate the efficacy of a change in process or practice. Longitudinal monitoring is also important for trend analysis and to provide an early warning of potential issues.

Contextualizing KPIs

Because of the many factors which can impact the success of fertility treatment and outcomes within the IVF laboratory, including scientific, clinical, environmental, and demographic factors, it can be helpful to try to put KPIs into context, particularly when comparing different laboratories or periods of time. At CARE Fertility, we have developed a simple tool which has proven helpful in contextualizing success rates based on patients being treated within a timeframe. [Figure 3.1](#) shows an example of the output of this tool. It uses four parameters: age of oocyte provider, AMH, oocyte number, and single embryo transfer to classify patients' prognoses from very poor through to very good. The proportion of patients in these simple categories enables us to anticipate and to compare results across multiple clinics in a different way, and to understand and quickly determine if results may not meet KPI targets in a particular time period. More often than not, a period where KPIs may not be met aligns with a period with a high proportion of patients being treated with very poor or poor prognosis, according to the criteria used in this tool. And this has already been predicted.

Using KPIs

It is important to consider the factors which make KPIs valuable in the IVF laboratory. They need to be measurable, reliable, and

trusted. Users should have confidence and belief in the quality of the data utilized for calculating these indicators of performance, which will enable their reproducibility and reliability. Use of expert consensus-championed PIs is wise, and enables access to carefully considered levels to benchmark against. As defined in the Vienna consensus for laboratory performance indicators (PIs), the high level, and most important indicators, are referred to as "key"—KPI; and these relate to the core activity in the IVF laboratory. Other indicators, referred to as PI, are helpful for scrutinizing specific areas of practice or process and, whilst they may not be assessed as frequently as KPI, accurate data should be collected to enable more detailed analytics, as needed. Reference indicators (RIs) can also be of use for providing a proxy indication of something, or for benchmarking between practitioners or laboratories, as these indicators relate to aspects that are outside of the laboratory and, as such, are less controlled or influenced.

With so many variables which cannot be controlled by the laboratory (e.g., patient clinical factors) or that the laboratory may have little influence over (e.g., medical practices), as described earlier, whilst somewhat challenging, it is important to ensure that laboratory KPIs are closely, and not tenuously, linked to laboratory activity. Large data sets can help minimize the impact of outliers, although many IVF laboratories may require long durations to collate sufficient numbers, by which time things may have changed.

Reference populations and KPI

Due to the impact that patient factors can have on laboratory outcomes relating to IVF and intracytoplasmic sperm injection (ICSI), along with clinical outcomes, when assessing KPIs, a reference population that excludes outliers and variables which can skew results is recommended. The following criteria for inclusion in the reference population were proposed by the ESHRE and Alpha expert consensus group:

- Oocytes from patients <40 years
- Autologous, fresh oocytes undergoing IVF or ICSI
- Fresh or frozen ejaculated sperm
- No preimplantation genetic testing (PGT)

In the interest of size of data set and considering current practice and outcomes, exclusions could be minimized by revising this list to include vitrified/warmed oocytes within the reference population; considering the age of the patient at the time of oocyte cryopreservation. This is because outcomes between thawed oocytes are widely reported to be similar to fresh oocytes.

Using a reference population for KPI monitoring gives a less heterogeneous set of data and therefore enables more reliable benchmarking of results across different laboratories, within a network, region, country, and beyond; providing that the reference population is agreed by all participants and the data is

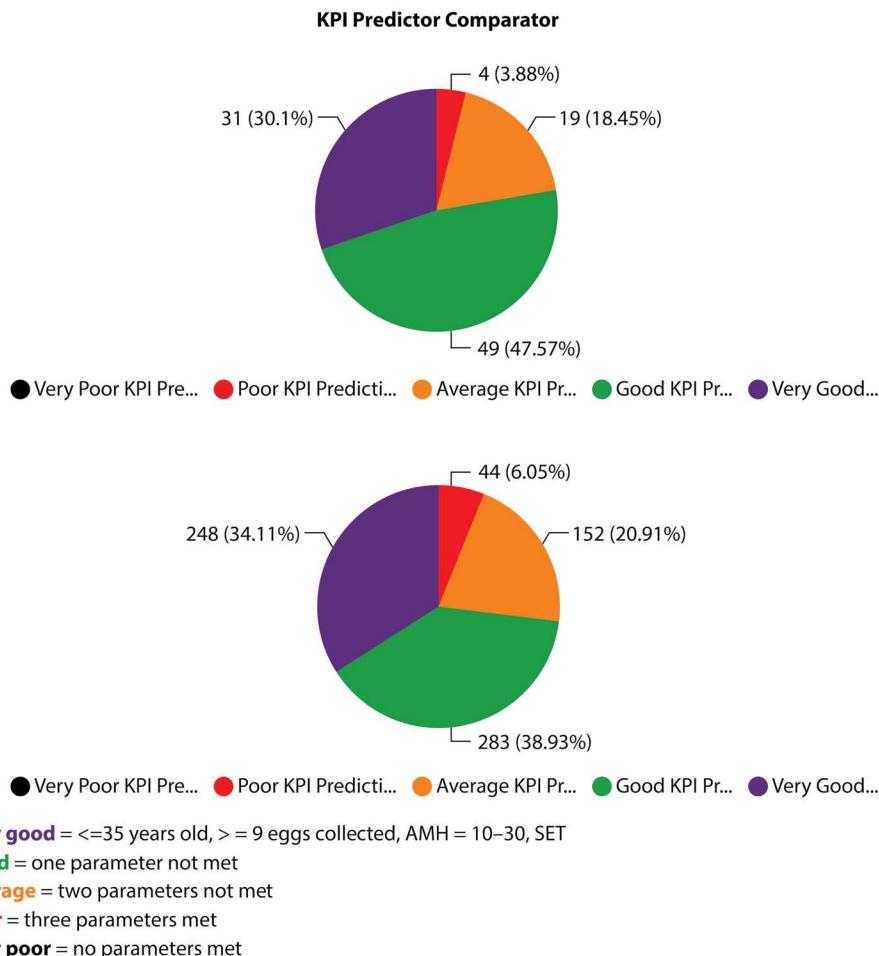


FIGURE 3.1 KPI prediction by simple patient factors.

recorded accurately. Once established, the reference population should be maintained and not revised regularly as this allows longitudinal monitoring and trend analysis to be undertaken.

Having a narrower reference population e.g., oocytes from patients <30 years, can be useful, but unless the clinic is conducting high numbers of treatments, the benefits can outweigh the drawbacks, such as the length of time it takes to undertake sufficient treatments for sample numbers to be of value.

Some clinics prefer to use several reference populations, one being “gold standard” patients. This population should have very good prognosis and may include patients undertaking their first IVF/ICSI treatment, include oocytes from young patients where the number of eggs may be within an “optimal” range, normal semen parameters and single embryo transfer, etc., for example.

Working within a network of clinics

The use of expert consensus guidelines can be an invaluable tool for laboratories to benchmark their own performance against reported industry norms or indicators. However, some laboratories may perform different proportions of complex cases and offer different treatments to other laboratories, and may not gain fully from generic KPIs.

Clinic groups, or networks, are becoming more common and one of the advantages for the laboratories within them is the facility for direct comparisons of KPI according to subpopulations of patients or treatment types, which may not be as straightforward to undertake with laboratories working with less consistency in practice.

Funnel plots are particularly useful in providing visual comparisons of multiple laboratories’ performances relating to specific KPIs whilst considering the number of treatments performed, and identifying where they sit within control limits or target values.

Figure 3.2 shows an example of a funnel plot for the CARE Fertility group when they were 10 clinics. During the period depicted, all were performing above the lower control limits. Each diamond represents a clinic plotted within a funnel according to the number of IVF treatments undertaken per year, and their individual fertilization rate for IVF, in this case.

Variation within one KPI: e.g. IVF fertilization rate

Some KPIs have several variations, and users may need to select which works best for them in their laboratory. Convention and

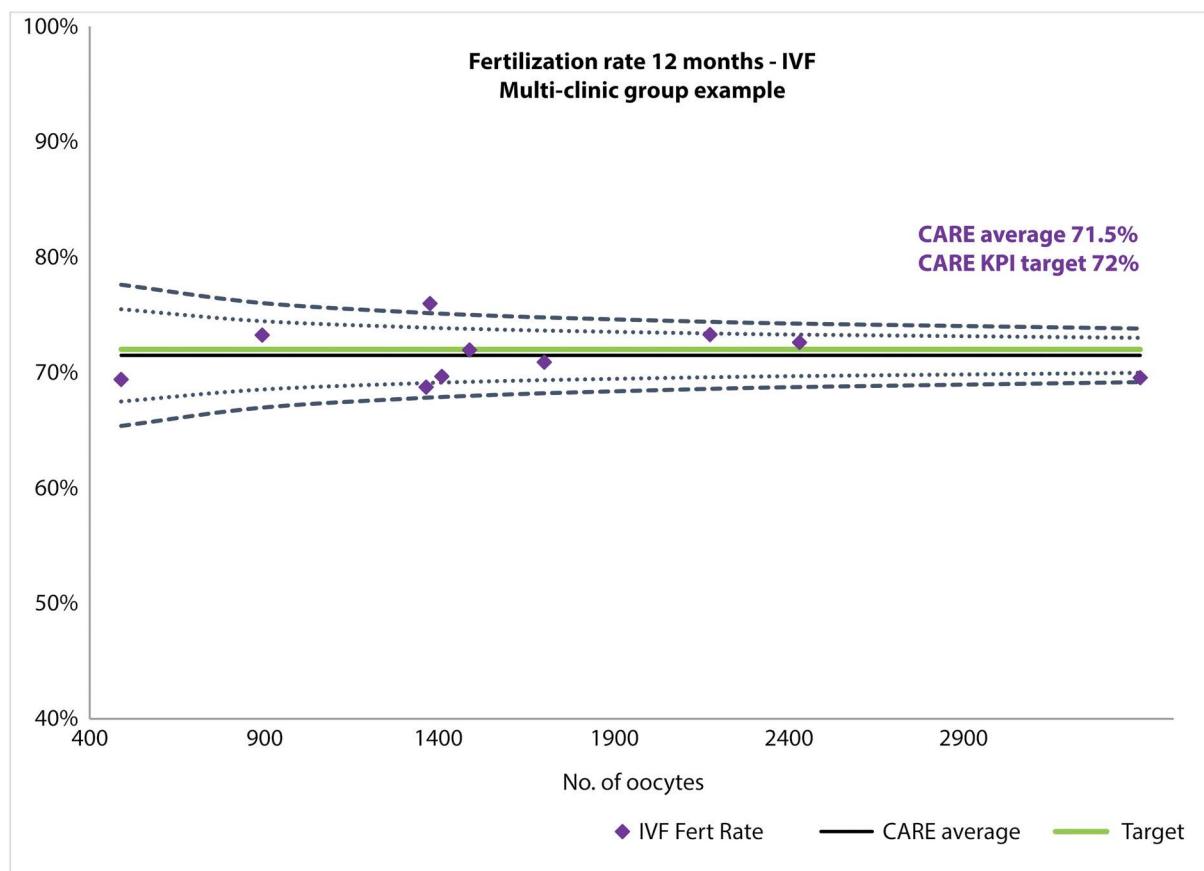


FIGURE 3.2 Funnel plot example for IVF fertilization rate.

expert consensus suggests that the IVF fertilization rate should be calculated as follows:

IVF normal fertilization rate (%) = number of oocytes with 2 pronuclei and 2 polar bodies divided by the number of cumulus oocyte complexes (COC) inseminated (multiplied by 100)

The consensus KPI values associated with this are a competency level of $\geq 60\%$ and a benchmark (aspirational target) of $\geq 75\%$ (Vienna consensus 2017).

This is a typical example of a KPI with several, justifiable alternatives. For example, the denominator in this KPI calculation may be impacted by clinical operating practice, which is often outside of the laboratory team's control, deeming it less useful as a KPI if clinical practice is not standardized. For example, follicular flushing, whilst generally not recommended or considered best practice, is still performed in some clinics, or by some practitioners. Flushing of smaller follicles, in particular, can result in the aspiration of less mature cumulus–oocyte complexes and therefore lower “IVF normal fertilization rate.” KPI selection, therefore, requires careful consideration and users should be mindful of the limitations of them.

The use of instinct for indicating

Unless updated very regularly, with large data, many KPIs will, by design, be somewhat outdated. Therefore, there remains a

place in the IVF lab for continuous scrutiny and challenge; and laboratory staff should remain mindful of the importance of their observational skills, experience, and instinct to detect early signs of potential issues in the laboratory.

In many IVF laboratories 20–30 years ago, there was a daily, audible indicator of quality! The computer-controlled freezers, with the noisy pressure pumps could be heard, often in the afternoons, demonstrating high-quality supernumerary embryos being cryopreserved. If this irritating but comforting noise wasn't heard, it was a useful indicator that embryo quality (at least that day) may be poor. In today's busy labs, with flexible working and increasingly varied role profiles, embryologists may have less continuity or spend less time within IVF laboratories to be able to make such sensitive and live observations to flag potential issues ahead of KPI generation.

Interdisciplinary team communication is vital to ensure scrutiny and early warnings. For example, if the ultrasonographer comments that, with the newly introduced gonadotropin, they have noted a different pattern of follicular growth, this should be recorded and an assessment of oocyte maturity and fertilization in the IVF laboratory may be brought forward in this case.

Another real-life example of the importance of observing and reporting the unusual in the IVF lab is the appearance of late vacuolation in the peri-compacting embryo. This may not be detected in core KPI, like blastulation rates, but may be an indication of osmolality deviations in the culture environment, and needs further scrutiny.

Broad shoulders in the IVF laboratory

Some IVF laboratories have a “broad-shoulders” approach to KPIs. Whilst this may seem unconventional and uncomfortable for some, the use of transparent and regular review of individual practitioners’ results, by laboratory process, can be highly effective in identifying and ensuring best practice and continuous improvement.

For example, at CARE Fertility, the results of each laboratory team member’s own results, for several outcome variables, are automatically and regularly generated and reviewed. For example:

- Fertilization rate by sperm preparation, oocyte recovery, cumulus removal, IVF, ICSI
- Implantation rate by embryo transfer
- Cryo-survival rate by oocyte/blastocyst vitrification and by warming
- Implantation rate by embryo warming

A similar approach is made for medical practitioners.

This system, if utilized, requires clear communication that this is undertaken together, in the interest of our patients and primarily to highlight best practices. It is also very important that everyone involved understands the high number of confounding factors within the IVF process and that these broad-shoulders

results are purely considered as potentially indicative and not absolute. If one practitioner is excelling in one area, for example, with 90% fertilization observed following their ICSI, then scrutiny of their practice may be warranted, along with coupling them up with the lower performing practitioners for observational and training sessions.

In general, it appears that the more attention that is given to the small details, and the more visible the IVF laboratory’s performance, the better the outcomes.

Published recommended KPIs: Are they still relevant?

A comprehensive and commonly used consensus report of an expert meeting on the development of assisted reproduction laboratory PIs was published in 2017 [1]. Twelve KPIs, five PIs, and two RIs were recommended for “fresh” IVF and ICSI treatments. Competency levels along with aspirational benchmarks were proposed for these 19 indicators. The KPIs are summarized and defined in [Figure 3.3](#).

For cryopreservation, the proceedings of an expert consensus meeting were published around a decade ago. It proposed 14 KPIs with benchmarks for cryopreservation.

With evolving clinical practice, and the associated increase in the proportion of single blastocyst transfer, pre-implantation

KPIs	✓ Competency	★ Benchmark	KPIs	✓ Competency	★ Benchmark
1. IVF Fertilization rate	≥60%	≥75%	7. Day 3 development rate	≥45%	≥70%
2. ICSI Fertilization rate	≥60	≥75	8. Day 5 development rate	≥40%	≥60%
3. Failed Fertilization rate	<5%	<5%	9. Successful biopsy/tubing rate	≥90%	≥95%
4. ICSI degeneration rate	≤10%	≤5%	10. Blastocyst cryo-survival rate	≥90%	≥99%
5. Day 2 cleavage rate	≥95%	≥99%	11. Cleavage stage implantation rate	≥25%	≥35%
6. Day 2 development rate	≥50%	≥80%	12. Blastocyst implantation rate	≥35%	≥60%

Definitions	
1. 2PN/COC inseminated	7. 8 cell stage embryos/2PN
2. 2PN/oocytes inseminated (ICSI)	8. Blastocysts/2PN
3. Cycles without 2PN/stimulated cycles	9. Amplified DNA/biopsied embryos
4. No. oocytes lysed/inseminated (ICSI)	10. Blastocysts intact/Blastocysts warmed
5. Cleaved embryos/2PN	11. Sacs (ultrasound detected)/cleavage embryos transferred
6. 4 cell stage embryos/2PN	12. Sacs (ultrasound detected)/blastocysts transferred

FIGURE 3.3 Vienna consensus reference indicators.

genetic testing for aneuploidy (PGT-A), and “freeze-all”—whereby embryos are not transferred fresh within the stimulated cycle but vitrified and warmed later for a “frozen embryo transfer” (FET)—several of the established KPIs may not be so relevant now. A number of recent publications consider this, and also ask whether KPIs are transferrable if patient treatment plans change e.g. from fresh to freeze-all [2, 3]. Due to these significant changes in blastocyst cryopreservation practice and results, the Vienna consensus group proposed a KPI for blastocyst cryo-survival.

Another KPI which was not included within the Vienna consensus but has recently been demonstrated to be useful, is day 5 usable blastocyst rate. KPIs such as this have the advantage over clinical outcome-related KPI in that they can be used to detect the efficacy of a controlled change, or raise alert to a negative trend. With the numerator in the KPI calculation being embryo number (as opposed to number of cycles/embryo transfers), this has the advantage of having the potential to detect KPI shifts in clinics with lower cycle volumes [4]. The Vienna consensus was critically appraised soon after its publication [5].

A recent publication explored the potential need for fine-tuning of the Vienna consensus according to female age. Interestingly, it concluded that most laboratory outcome measures were reliable irrespective of female age. However, KPIs relating to extended embryo culture should be fine-tuned to consider female age, due to good quality blastocyst rate being independently associated with it [6].

The future of KPIs

Across IVF laboratories worldwide, there are varied approaches to the use of KPIs, both in the value these are given within the IVF laboratory and in the complexity and detail within the approach, which ranges from basic, *ad hoc* use to highly sophisticated, automated systems.

With a movement toward digitalization and automation within IVF clinics and laboratories generally, KPI monitoring of the future is likely to become less laborious, rapid and sensitive, with the potential to incorporate large and live data sets.

Several commercially available tools already exist to enable laboratory data to be processed semi-automatically, with the facility for laboratory staff to customize reports, graphs, dashboards, and functions. These may use CSV files or other formats. These tools allow users to switch between parameters and subpopulations, and can provide side-by-side comparisons.

As data sets become increasingly detailed and digitalized, and data storage and transfer more flexible, artificial intelligence is

likely to be increasingly utilized, along with deep learning, to detect and anticipate IVF laboratory KPIs [7].

References

- ESHRE Special Interest Group of Embryology, Alpha Scientists in Reproductive Medicine. The Vienna consensus: Report of an expert meeting on the development of art laboratory performance indicators, Human Reproduction Open. 2017;2017(2):hox011. <https://doi.org/10.1093/hropen/hox011>
- Fabozzi G, Cimadomo D, Maggiulli R, Vaiarelli A, Ubaldi FM, Rienzi L. Which key performance indicators are most effective in evaluating and managing an *in vitro* fertilization laboratory? Fertil Steril. 2020;114(1):9–15. doi: [10.1016/j.fertnstert.2020.04.054](https://doi.org/10.1016/j.fertnstert.2020.04.054).
- Wang HT, Hong PP, Li HY, Zhou W, Li T. Use of a new set of key performance indicators for evaluating the performance of an *in vitro* fertilization laboratory in which blastocyst culture and the freeze-all strategy are the primary treatment in patients with *in vitro* fertilization. J Int Med Res. 2021;49(9):3000605211044364. doi: [10.1177/03000605211044364](https://doi.org/10.1177/03000605211044364).
- Hammond ER, Morbeck DE. Tracking quality: Can embryology key performance indicators be used to identify clinically relevant shifts in pregnancy rate? Hum Reprod. 2019;34(1):37–43. doi: [10.1093/humrep/dey349](https://doi.org/10.1093/humrep/dey349). Erratum in: Hum Reprod. 2019 Apr 1;34(4):780. PMID: 30517659.
- Lopez-Regalado ML, Martínez-Granados L, González-Utor A, Ortiz N, Iglesias M, Arroyo M, Castilla JA. Critical appraisal of the Vienna consensus: Performance indicators for assisted reproductive technology laboratories. Reprod BioMed Online. 2018;37(2):128–32.
- Zacà C, Coticchio G, Vigiliano V, Lagalla C, Nadalini M, Tarozzi N, Borini A. Fine-tuning IVF laboratory key performance indicators of the Vienna consensus according to female age. J Assisted Reprod Genet. 2022 Apr;39(4):945–52.
- Bormann CL, Curchoe CL, Thirumalaraju P, Kanakasabapathy MK, Gupta R, Pooniwala R, Kandula H, Souter I, Dimitriadis I, Shafiee H. Deep learning early warning system for embryo culture conditions and embryologist performance in the ART laboratory. J Assist Reprod Genet. 2021;38(7):1641–46. doi: [10.1007/s10815-021-02198-x](https://doi.org/10.1007/s10815-021-02198-x).

Further reading

ESHRE Clinic PI Working Group, Vlaisavljevic V, Apter S, Capalbo A, D'Angelo A, Giarolli L, Griesinger G, Kolibianakis EM, Lainas G, Mardesic T, Motrenko T, Pelkonen S, Romualdi D, Vermeulen N, Tillemans K. The Maribor consensus: Report of an expert meeting on the development of performance indicators for clinical practice in ART. Hum Reprod Open. 2021;2021(3):hoab022. doi: [10.1093/hropen/hoab022](https://doi.org/10.1093/hropen/hoab022).

4

QC IN THE CLOUDS *Digitizing Quality Control*

Giles Anthony Palmer

The most profound technologies are those that disappear. They weave themselves in the fabric of everyday life until they are indistinguishable from it.

Mark Weiser, Computer Scientist [1]

The rise of quality control to meet the clouds

Quality management is the cornerstone of modern clinical embryology. No longer a novel idea, a long-established doctrine that states that a successful *in vitro* fertilization (IVF) laboratory requires a high level of quality control, ensuring constant improvements by a continuing cycle of implementation, evaluation, reflection, and corrective action [2].

In fact, it is hard to remember when quality control in the IVF laboratory was not so fiercely defended. Nowadays it is either highly recommended or mandatory [3–6], but in the early 1980s, after the birth of the first child following IVF, standards relied on self-motivation; gradually national bodies and societies developed quality guidelines and published best practice guidelines featuring the necessity of having a total quality management system [7, 8].

One of the first to address quality control *per se* was Lynette Scott in 1993, where many aspects of embryo culture were under scrutiny using a mouse embryo bioassay to look at the effects of such variables as type of water, media composition, contact materials, and incubators conditions [9]. Another more clinically oriented study by Matson in 1998 described mechanisms of internal quality control, external quality assurance, and audits as being useful tools for monitoring laboratory performance, cementing the idea in the psyche of the clinical embryologist that “moving the work from being a subjective art form to an objective science” was beneficial to IVF success [10].

Despite this mainstream adoption, the tools of quality control have not progressed greatly in several decades. Most clinics use antiquated ways of recording laboratory data with only a cursory contemplation of the results [11].

Why in this electronic age is data often buried in folders and binders with analysis rare and only examined at times of clinic inspections?

One reason is perhaps that it has not become standardized. Today, the practice of quality control in laboratories across the world can *at the very least* be described as heterogeneous. Knowledge of what each laboratory undertakes to pursue its version of quality excellence is unknown and is based loosely on the legacy of former laboratory managers and the latest opinions on optimum laboratory conditions and manufacturers’ recommendations.

The ever-evolving IVF laboratory has a myriad of parameters to check, and with each new addition to the laboratory, every latest piece of equipment brings its own quality control challenges.

What are the factors that we should know intrinsically about our own labs in the process of quality control?

The evaporation rate of our dewars? Or the gas consumption rate of our incubators? The optimum temperature for the heated stage of the intracytoplasmic sperm injection (ICSI) rig or even the best temperature on the warmed surface where the oocyte collection is performed?

We are told that everything in the laboratory is important [12] but to what appropriate level should it be monitored? What are the optimum environmental conditions for the lab? Which are the vitally important checkpoints, and which are superfluous?

To answer these questions, we must go to the root of the problem; quality control is time-consuming and the manner to how it is generally performed can interfere with workflow.

Collection and serious analysis of laboratory data is still hindered by using pen and paper. Any attempt to seriously interpret this data would require at least transcribing to an electronic worksheet but entails a cumbersome step which may introduce errors [13]. In contrast, the use of a mobile application (app) using cloud computing would reduce paperwork and provide a modern, convenient, and insightful way to look at data.

Cloud computing has changed the face of how businesses handle information technology since the mid-2000s [14]. It delivers to any clinic or company computing as a service and, with no in-house servers, provides a network of multiple computers and servers connected to each other over the internet. These services are monitored closely so problems are fixed whenever they occur, and users do not have to worry about maintenance and system upgrades. Cloud computing allows data to be accessed anywhere and creates a remote way of monitoring the periodic quality control data.

Quality control using the cloud has been present for many years in other industries and is an important part of good practices used in fields such as aerospace and defence, pharmaceuticals, manufacturing, electronics, and the automotive sector (Advantive Inc., USA). Stakeholders use this service as a proactive tool to improve product performance where non-compliance, similar to the IVF industry, may lead to fines, operational shutdowns, and stern legal intervention.

Quality control reporting has at its very core the monitoring of periodic drift. Drift may seriously influence the outcome and can signal a decline in equipment performance and well-being if left unchecked. Wadewale and Desai [15] describe six basic types of drifts and if not electronically recorded they can be difficult to recognize, subtle changes may be overlooked, and abrupt changes may be either missed or fleetingly disregarded. Using electronic means allows a way to precisely classify the data, reflect, and adapt to such changes quicker, whether these are sudden, incremental, or gradual deviations, recurring, sudden, or simply “noise” (Figure 4.1).

The “new normal” of work-from-home, work-from-anywhere, flexible hours, and our new mindset [16] make cloud computing

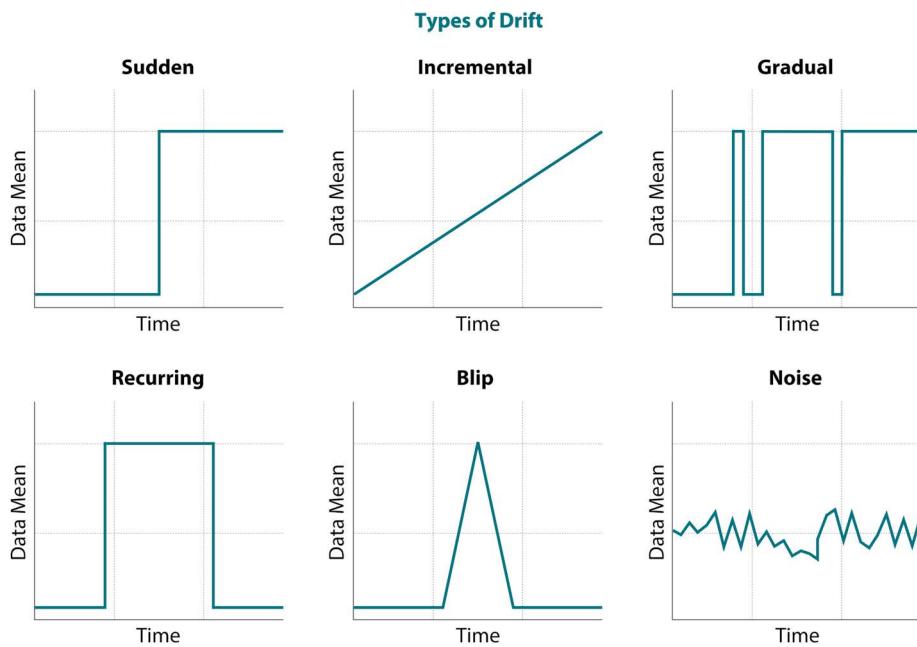


FIGURE 4.1 Types of drift. (Adapted from [15] with permission.)

a prerequisite for following laboratory quality control remotely using timely advances in technology.

One such cloud-based app specially design for the IVF laboratory is Reflections (IVFQC, Althea Science, USA). One of a suite of apps that aims to improve recording and action on quality control data. Accessible from wherever there is an internet connection, it provides the clinic with an inspection-ready electronic ledger of lab logs, recorded parameters, and statistical analysis. Whether data is in paper or electronic form, the records must be analyzed, and this cloud-based application provides a platform to add instrument parameters and perform statistical and fluctuation reports in a clear and concise way (Figure 4.2).

Specifically designed for the IVF laboratory, and accessible by a personal computer (PC), smartphone, or tablet, the format of entering data can be fully customizable to mimic any previous tabulations that were transcribed using paper and clipboard.

In the absence of standardized guidelines and in the void of global information surrounding the quality control practices in the assisted reproductive technology (ART) laboratory this author reported the finding from a study looking at the habits of users of this app [17] in a global setting. This novel “call-to-action” [11] involved a study of 36 laboratories in 12 different countries conducted to assess differences and similarities between laboratories using this adaptable cloud-based quality control app.

Data from equipment and quality control recordings were grouped into domains, according to their function in the laboratory, such as “incubators,” “air quality,” “heated stages,” and “cryo-storage vessels,” and the corresponding data was analyzed both individually and as a whole.

It was no surprise in the results that the embryologist attention was mainly focused on the incubators, still the “work horse” of the lab where 50% of all data entries were attributed to this domain, followed jointly by warming stages and cryo-room readings at 11%; 9% of all data points were used for checklists where compliance to certain protocols were recorded electronically on the app.

The study showed the differing global habits and different ranges that clinics accepted for minimum and maximum thresholds such as incubator O₂ and CO₂ concentrations and temperature values in instruments such as warming stages, heated surfaces, refrigerators, and freezers.

Regarding recording daily measurements, the study showed that the participating laboratories all measure incubator parameters, while 91% recorded warming stages and 41% the medical gas manifold. Interestingly, only 36% of clinics manually monitored dewars, despite the recommendations of visual observations being a minimum requirement in risk management of cryo-storage of reproductive tissue [18].

The intensity of data collection also varied between the clinics. To quantify this disparity a surrogate indicator of quality control diligence, the MAD score (mean average data score), was formulated. It highlights the most conscientious clinics by using the number of data entries per day divided by the number of instruments monitored (Figure 4.3). Great variation was observed between the clinics.

The higher the score, the more manual quality control readings were being conducted regardless of the size and volume of work performed by the clinic. If daily checks are to be standardized it would be fair to say that the MAD score would be similar.

It remains to be seen if the laboratories with a higher MAD score enjoy a higher success rate, but, in a follow up study, authors noted that clinics display clear differences in reporting habits, and clinics that have a higher MAD score are in regions which have a rigorous regulatory body [19].

Although equipment in many laboratories is monitored in real time and continuously logged, the frequency of manual inspections should not be underestimated, and at least a daily visual check of equipment needs to be performed [20]. Often delegated to more junior members of the laboratory team, the daily log can help tremendously the young scientists’ orientation into clinical embryology and induction into the requirements and trouble shooting skills which are essential traits of the profession [21].

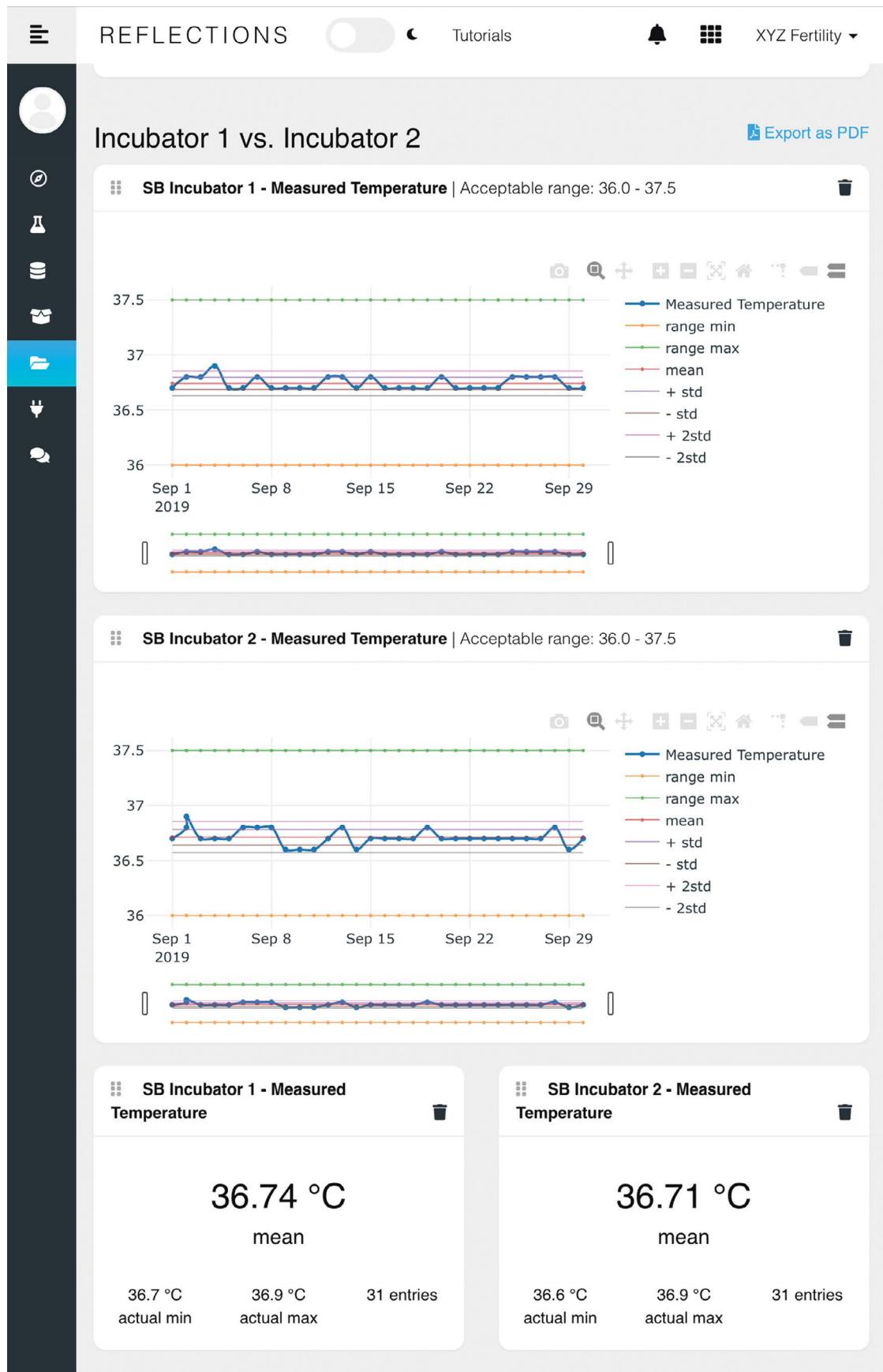


FIGURE 4.2 A typical screen display showing temperature input over time and statistical data in a laboratory setting.

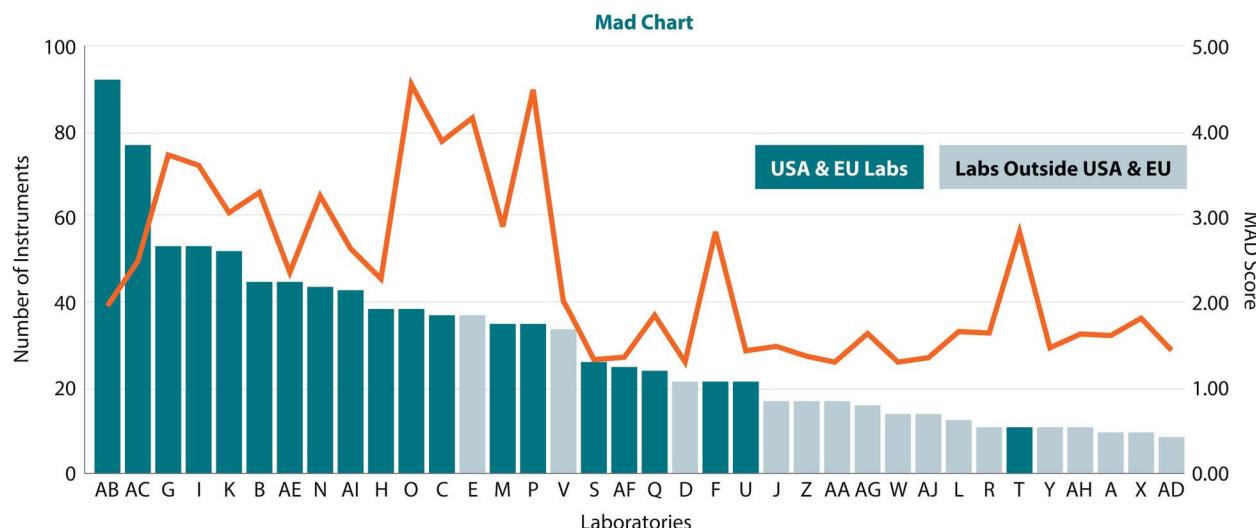


FIGURE 4.3 The highest MAD scores originated from countries where traditionally the IVF health sector had been more rigorously governed; of the top 50% of clinics in this study in relation to the MAD score, 89% were located in Europe or North America. (Adapted from [19].)

Finally, the daily duty of performing an electronic “lab log” can safeguard against data entry being inadvertently missed or ignored. Alarms and reminders may alert the embryologist of any deviation from the desired result.

Simply put, cloud computing gives the broadest access to the data; allowing more senior embryologists to manage single or multiple sites synchronously; following task completion by their staff and reviewing data entry from afar.

When clouds appear like towers

Few would disagree that quality control has made the practices in the IVF laboratory safer, continually on a path of persistent improvement through corrective and preventative actions, but it remains to demonstrate improving clinical outcome. Although cloud computing gives immediate access to statistical and graphical interpretation of laboratory data it remains shielded in “silos,” departmentalized, and fragmented from other data streams in the IVF clinic.

A question remains: Can any of these measured parameters be linked with clinical outcome? Notwithstanding there being more than 200 cofounders that can impact IVF outcome [22], every step of the patient’s journey is now traceable through electronic medical records; from first rendezvous, diagnosis, and treatment planning to tracking and tracing gametes and embryos during their *in vitro* residency and subsequent fate. Surely linking this data to quality control data is the first step to resolving this problem.

Despite the fear of leaving a clinic vulnerable and exposed to litigation in the event of an adverse condition, each clinic with a robust quality control system should not be duly concerned. At last, we should be able to use all the data from a clinic like “business intelligence” where insights into performance, goals, and operations can be analyzed, forming data-driven decisions for improving success.

While many innovations in our field have been adopted quickly into the IVF laboratory, there appears to be some resistance to change concerning data processing and digitization of quality control data. Responsibilities of the clinical embryologist have

changed in recent years, giving rise to increasing screen time as growing demands on regulations and documentation changes the daily routine to include extensive administrable duties [23]. Perhaps the duties entailed in quality control are unpopular tasks seen as inconveniences and time-consuming.

To avoid the dissatisfaction that has been cited in similar circumstances, with clinicians asserting that electronic clerical duties are overwhelming, compete with patient “facetime” [24, 25], and lead to burnout [26], we must work smarter to avoid these failing. Indeed, embracing this flurry of interest in automation, AI and big data may be a necessary watershed in the role of the embryologist where technical skills might become less important while a growing leaning toward information technology will prove advantageous [27].

The digital lab

We have always belonged to an industry moving at a fast pace incorporating advances from various areas of science in its 40-plus years of development [28] with a penchant toward refinement with the promise of automation [29, 30] and recently the use of big data and AI [31] to make our decisions more precise, more accurate, and repeatable. Until this goal is reached, clinical embryology remains very different to other medical labs; it is largely manual in nature, requiring good hand–eye coordination with precise, delicate skills taught through a process of practise and competence assessments [32].

However, the workplace itself is changing, with veteran members leaving the workplace giving rise to new generations of embryologist better equipped to deal with the legacy of this expansion of information technology in our industry [33].

“Liquid expectations,” the expectations that the user experience (viz. young embryologists and patients alike) that is enjoyed in other technologically advanced activities like online booking, streaming services, e-commerce, and logistics [34], may very well seep into this industry, heralding a quicker implementation of new digital innovations.

Indeed, more and more assignments are heading for the cloud—pushing everything to be accessible anywhere and anytime.

The increasing use of the smartphone has created better opportunities of engagement for patients and ART professionals alike [35], seeing that a transparent, compliant electronic audit trail is preserved. Telemedicine is now widespread within the health services [36, 37] and is benefiting from this integration accordingly. Patients can feel more engaged using an app such as the “SART mobile,” launched by the Society for Assisted Reproductive Technology [38], which connects patients with resources and information about infertility in the United States, while Salve (Salve Technologies Limited, UK) uses a cloud-based “patient engagement platform” to streamline client workload to improve the patient experience.

Even home sperm assessments through mobile devices are being explored, measuring accurately and precisely motile sperm concentration all from a convenient smartphone [39].

For the embryologist, too, there are apps such as ART Compass LLC and the Proficient Lab LLC that use cloud computing for many aspects of laboratory quality management that were previously confined to the PC. Mobile access to training records, benchmarking, personal KPIs, and more are pushed to the phone or tablet, eliminating paperwork, optimizes training and competency records while keeping the clinical embryologist engaged in their person development and obligations.

As an increasing number of companies are offering novel products to the ART industry, compatibility and connectivity between various appliances and instruments has becomes a concern. This synergy has been accelerated through the use of an application programming interphase (API) allowing any two systems to link cloud services together. Like an “electronic handshake” these secure keys form an important development to facilitate a more extensive service where various desirable features from different companies can be easily accessible remotely on a single device.

One area where APIs are crucial is in the monitoring of crucial IVF laboratory equipment 24/7. Mandatory in many countries [4, 40], we require a modern way of following mission critical streaming data. While many systems rely on a relay system of multiple points of telecommunications or radio frequency transmitters to alert clinic staff, there are now available easy-to-install smart devices. These sensors can send real-time monitoring data direct to the cloud without intermediate servers via an API, completely bypassing any traditional networks and providing a system without routers, servers, or access points (PharmaWatch™, American Pharma Technologies LLC, USA).

Unavoidable risks in cryo-storage, too, may be reduced through several recent advances concerning the safety of gametes and embryos stored onsite. There has been a call for reckoning [41] following catastrophic events in 2018 that resulted in the loss of thousands of cryo-stored oocytes and embryos [42, 43]. Tank surveillance through the cloud, especially using thermal imaging (Cryosentinel LLC), offers a quick visual interpretation of dewar integrity and may well appease current fears about reaction times in the event of a catastrophic vacuum failure [44].

Also, new technologies may aid current short comings in the chain of custody. Quality control for decades has been assisted by the traceability and accountability given by electronically witnessing laboratory events. This “benchtop tagging” using either barcode or RFID tagged samples (IMT Matcher™, RI Witness™) assures compliance with protocols and reduces mismatch errors through proximity alerts in the embryology lab; and it has been absent from the cryo-room.

However, location tracking using thermo-tolerate RFID tags either submerged in liquid nitrogen itself [45] or embedded in

a capsule [46] can now locate samples from within the dewar, and with the use of an automated storage system (TMRW Life Sciences Inc) can alleviate the need for customary inspections and audits, greatly improving frozen sample management through novel software and the use of robotics.

Tracking samples in transit would complete the “cold-chain-of-custody” using a specialized dry shipper dewar where transportation of frozen gametes and embryos can be closely scrutinized using cloud computing. Already, conditional monitoring of many metrics other than just core temperature such as orientation, proximity, and shock, can be relayed to and from the cloud (SmartPak™, Cryoport Systems, LLC), mitigating risk and providing assurance for clinic and patient alike to the correct transport conditions and precise location of the precious cargo.

Since introduction in the mid-1990s, mobile phones have become firmly embedded in our society. The advent of 3G in mid-2001 offered mobile data handling capabilities which rapidly diffused [47], and the recent emergence of 5G technology will bring major improvements to cloud-computing services with low to zero latency and connections to devices, not just phones.

The evolution of the mobile technology has progressed from a simple communication device to a ubiquitous tool for data analysis [48], opening up possibilities of quick and easy access to laboratory quality data at our fingertips, but at some cost. . . .

Dark clouds

Increased access to clinic data does have its drawbacks. Safety of data storage is clearly paramount, and many countries have strict guidance for the transmission of data both within the walls of medical establishments and in the clouds.

Such is the case in the United States, where the Health Insurance Portability and Accountability Act (HIPAA) is a series of federal regulatory standards that outline the lawful use and disclosure of health information. It consists of three major components—privacy, security, and breach notification rules. All data usage must be HIPAA compliant [49].

Similar strict laws exist in Canada that govern personal and identifying data with the Personal Information Protection and Electronic Documents Act (PIPEDA) [50]; in Australia with the Privacy Act (Privacy Act), both recently amended to internet-based data handling [51]; and the General Data Protection Regulation (GDPR), a regime of personal data protection requirements adopted by the European Parliament [52]. Security and privacy with cloud computing remains a constant battle, with many services using two-factor authentication and end-to-end encryption (familiar to users of mobile banking services), resulting in a more secure way to store and procure data, but the industry must always be watchful.

Cybercrime flourished with the increase of home/remote use of systems in the light of the 2020 COVID-19 pandemic [53], and robust measures must be in place to prevent malicious access of criminal activities, ensuring clinics stay vigilant to cybercrime, and in particular ransomware attacks, which has led to debilitating consequences in several clinics worldwide [54].

Serious consequences ensue for any breach of confidentiality, and whereby most security breaches happen as a result of staff oversight, not familiar with data security, it is recommended regular data awareness courses would help the “end users” understand their obligations learning data handling etiquette [55] and remaining being vigilant to digital threats.

We may be becoming more connected as a society, but because of security issues, data fragmentation is a major problem that

prevents countries and organizations from sharing information. This may be solved by blockchain technology: using the cloud, or rather multiple networks within clouds, information packages can be uniquely tagged “block by block” onto the existing data. This creates a chain of information, an immutable ledger [56], that can be tracked and verified floating in the ether. Most recognized for its use in cryptocurrencies, this recent development has opened opportunities for the healthcare sector, offering an effective way of exchanging data and research material across health systems and even borders [57].

Finally, the system cannot “go down.” When relying on a third party to manage your data handling needs business continuity must be maintained at all times. This is being addressed by large cloud-computing providers who consistently back up data and employ redundancy within their electronic architecture to ensure that an individual failure has an immediate fall-back system. Additionally, “cross region deployment” of cloud servers scattered in different geographic areas and back-up “warm standby” servers aim to produce a “fault-tolerant service” [58].

QC in the fog

Sensors are getting smaller. Micro-electro-mechanical systems (MEMS) have enabled simple and inexpensive data collection in smart devices with low power usage, facilitating the emergence of networks of interconnecting devices in what has been described as the “internet of things” (IoT) [59].

This “ecosystem” of electronic devices where network connectivity and computing capability extends to objects, sensors, and everyday items not normally considered computers, allows these devices to generate, exchange, and consume data with minimal human intervention.

The internet of people becomes the internet of things and would not be possible without harnessing cloud computing fuelled by the development of the mobile network standard 5G [60, 61] offering super-fast connectivity.

According to Cisco Systems, Inc. there were 7.6 billion active IoT devices in 2020, a figure which will grow to 24.1 billion by 2030 [62]. At present, this technology is most visible in “smart homes” where home appliances are monitored and controlled via the world wide web [63, 64], but is rapidly being deployed in many other domains such as intelligent grids, waste management, farming, and energy management. There is increasing interest from healthcare markets [65] with a tremendous potential of IoT to improve patient safety, staff satisfaction, and operational efficiency.

The greatest body of work has been done with diabetes sufferers, with several applications using IoT smart insulin pens as a continuous glucose monitoring device preventing hyperglycaemia and hypoglycaemia [66].

Other “smart devices” are being trialled for the treatment of such conditions as asthma [67] and Parkinson’s disease [68]. Intelligent medicine packaging using embedded sensors and minute RFID tags can shadow patient compliance to drug administration at home monitoring when the package seal has been opened [69].

Familiar to smart watch owners and “life loggers” there are many wearables bordering on being called a medical device. Interacting with skin directly or through clothing, these devices can aid health and well-being; many patents now exist to measure specific human physiological parameters such as heart rate, respiration rate, and blood pressure [70].

Similarly, haptic or touch technology applies the forces of pressure or vibration to interface with the operator. This “force feedback” can be most recognized in gaming consoles and has been

present on wristwatches and health monitors in the form of tapping since 2015 [71].

Indeed, Amazon has patented a haptic wristband to steer store-room employees to the correct inventory and alert them if their package is incorrect [72].

Could this *new* sensory perception guide embryologists in their operations in the IVF laboratory and prevent mistakes?

It would not be a large stretch of the imagination if these new devices could log our activities around the IVF laboratory, tracking our movements and monitoring our equipment, offering complete transparency?

One study describes quality control in an IVF setting using a network of well-positioned IoT sensors measuring real-time key environmental parameters such as temperature, humidity, and volatile organic compounds (VOCs) content [73].

IoT, together with AI, is already used in quality control systems to maintain industrial machines and can analyse drift and predictive maintenance. The system can be further trained to predict the remaining useful life of the machine before it requires maintenance or replacement [74].

Equally, we may be able to harness this tech to detect changes in our daily routine and act as an early warning system, monitoring performance, self-diagnosing a fault, and sending for an engineer!

The ideal place to analyse and act on most IoT data is near to those very devices, and to this aim, “fog computing” has been developed. Fog in nature is low-forming cloud, and this phrase, coined by Cisco Systems, Inc. in 2015 [75], denotes a decentralized computer system consisting of “nodes” capable of performing both networking and computational operations at the same time that is closer to both the ground level and the user.

Fog computing promises low latency, as these nodes are closer to the user and can provide instant responses, require little bandwidth owing to the pieces of information are aggregated at different points along the network with no loss of connection.

High security is assured by the huge network of nodes in a complex distributed system with the possibility of blockchain security [76].

In its classical usage, quality control in the IVF laboratory is simple documentation to show that equipment and instruments are functioning within a predetermined range, but using IoT and wearables we could move toward a passive method of data collection leading to complete transparency of all procedures in the lab.

What started with the adoption of technological solutions for electronic witnessing in the early to mid-2000s [77] could now lead to a recording system finally not interfering with the workflow to create big data on everything connected to the lab!

As we draw closer to “ubiquitous computing,” first described by Mark Weiser [78], where we will have access to computing anywhere and in any situation, we rely on the technology of cloud computing to deliver us fast, reliable, and secure computing from any location.

In our own setting, a mix of mobile telephone applications, continuous monitoring systems safeguarding critical equipment, numerous IoT devices, and wearables could track and monitor events and conditions around the IVF laboratory. Precision timing of laboratory events, compliance to protocols, staff competency, and equipment monitoring could all be effortlessly recorded and monitored. High tech, low impact on workflow, these innovations could change the way we view quality control data collection in a “smart lab” in the near future (Figure 4.4).

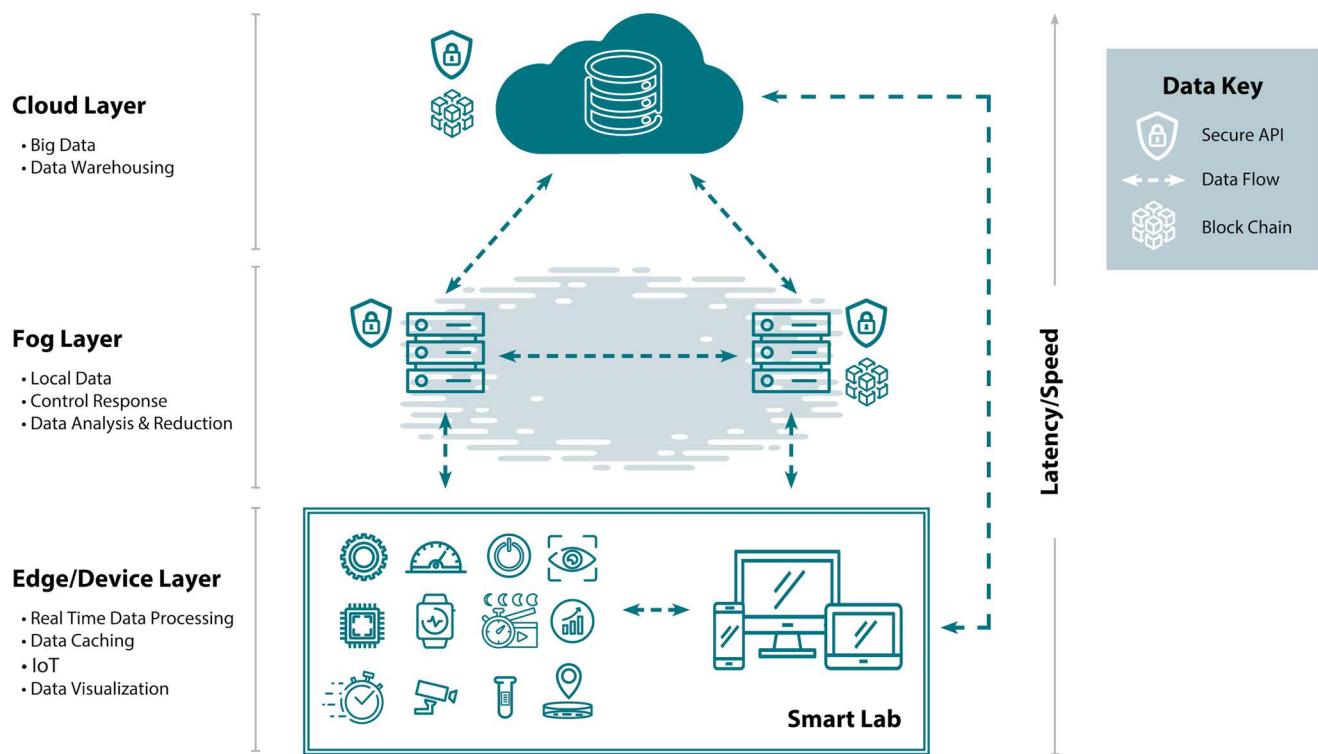


FIGURE 4.4 Smart lab.

As processes in the ART laboratory have constantly been improved and refined with the implementation of quality management, it is time to realize that data can now be processed with minimal effort and inconvenience with the maximum computational power using existing cloud services.

Quality control in the IVF laboratory can surely only benefit from a more digital integration and a more active uptake around the world by embryologists; it only remains to be seen if this forecast of new technologies can give a brighter outlook into the way we perform quality control—giving this cloud a silver lining.

References

1. Weiser M. The computer for the 21st century. *Sci Am.* 1991 September;265(3):94–104.
2. Mortimer ST, Mortimer D. Quality and Risk Management in the IVF Laboratory. Cambridge, UK: Cambridge University Press, 2015.
3. HFEA. 23. The quality management system. Code of Practice, 9th Edition, pp. 244, 2021.
4. U.S. Department of Health and Human Services, Centers for Medicare and Medicaid Services. Medicare, Medicaid and CLIA programs; laboratory requirements relating to quality systems and certain personnel qualifications. Final rule. 42 CFR 493. Fed Regist 68:3640–3714; 2021.
5. Canadian Fertility & Andrology Society. Competency Guidelines for ART Laboratory Professionals in Canada. 2022. https://cfas.ca/ART_Lab_Competency_Guidelines.html.
6. Reproductive Technology Accreditation Committee of the Fertility Society of Australia and New Zealand. Records of QC validation, maintenance and service of equipment including the frequency of testing. 2022. <https://www.fertilitysociety.com.au/wp-content/uploads/20211124-RTAC-ANZ-COP.pdf>.
7. Practice Committee of the American Society for Reproductive Medicine, Practice Committee of the Society for Assisted Reproductive Technology. Revised minimum standards for practices offering assisted reproductive technologies: A committee opinion. *Fertil Steril.* 2014;102(3):682–6.
8. De los Santos MJ, Apter S, Coticchio G, Debrock S, Lundin K, Plancha CE, Prados F, Rienzi L, Verheyen G, Woodward B, Vermeulen N, ESHRE Guideline Group on Good Practice in IVF Labs et al. Revised Guidelines for good practice in IVF laboratories. *Hum Reprod.* 2016 Apr;31(4):685–6. doi: [10.1093/humrep/dew016](https://doi.org/10.1093/humrep/dew016).
9. Scott LF, Sundaram SG, Smith S. The relevance and use of mouse embryo bioassays for quality control in an assisted reproductive technology program. *Fertil Steril.* 1993;60(3):559–68.
10. Matson PL. Internal quality control and external quality assurance in the IVF laboratory. *Human Reprod.* 1998;13 (Suppl_4):156–65.
11. Gardner DK, Pool TB. Contemporary quality control in the IVF laboratory: Heading to the cloud? *Reprod Biomed Online.* 2019;39(1):1–2.
12. Group CC. There is only one thing that is truly important in an IVF laboratory: everything! Cairo consensus guidelines on IVF culture conditions. *Reprod Biomed Online.* 2020;40(1):33–60.
13. Mays JA, Mathias PC. Measuring the rate of manual transcription error in outpatient point-of-care testing. *J Am Med Inform Assoc.* 2019;26(3):269–72.
14. Kratzke N. A brief history of cloud application architectures. *Appl Sci.* 2018;8(8):1368.
15. Wadewale K, Desai S. Survey on method of drift detection and classification for time varying data set. *Int Res J Eng Technol.* 2015;2(9):709–13.
16. Popovici V, Popovici AL. Remote work revolution: Current opportunities and challenges for organizations. *Ovidius Univ Ann Econ Sci Ser.* 2020;20:468–72.

17. Palmer GA, Kratka C, Szwetecz S, Fiser G, Fiser S, Sanders C, Tomkin G, Szwetecz MA, Cohen J. Comparison of 36 assisted reproduction laboratories monitoring environmental conditions and instrument parameters using the same quality-control application. *Reprod Biomed Online*. 2019;39(1):63–74.
18. Pomeroy KO, Reed ML, LoManto B, Harris SG, Hazelrigg WB, Kell DA. Cryostorage tank failures: Temperature and volume loss over time after induced failure by removal of insulative vacuum. *J Assist Reprod Genet*. 2019;36(11):2271–8.
19. Palmer GA, Kratka C, Szwetecz S, Fiser G, Fiser S, Sanders C, Tomkin G, Szwetecz MA, Cohen J. Instrumentation Monitoring Diligence in IVF Laboratories Worldwide. Barcelona: ISIVF, pp. 24–26, Oct 2019.
20. Hreinnson J, Quality management. In A Practical Guide to Setting Up an IVF Lab, Embryo Culture Systems and Running the Unit. Vargese A, Sjöblom P, Jayaprakasan K. (eds.). New Delhi: Jaypee Publishers, pp. 87–93, 2013.
21. Choucair F, Younis N, Hourani A. The value of the modern embryologist to a successful IVF system: Revisiting an age-old question. *Middle East Fertil Soc J*. 2021;26(1):1–6.
22. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. In *Embryo Culture*. Totowa, NJ: Humana Press, pp. 367–86, 2012.
23. Campbell A, Cohen J, Ivani K, Morbeck D, Palmer G, Mortimer S. The in vitro fertilization laboratory: Teamwork and teaming. *Fertil Steril*. 2022 Jan;117(1):27–32.
24. Flanagan ME, Militello LG, Rattray NA, Cottingham AH, Frankel RM. The thrill is gone: Burdensome electronic documentation takes its toll on physicians' time and attention. *J Gen Intern Med*. 2019;34(7):1096–7.
25. Payne TH. EHR-related alert fatigue: Minimal progress to date, but much more can be done. *BMJ Qual Saf*. 2019;28(1):1–2.
26. Melnick ER, Dyrbye LN, Sinsky CA, Trockel M, West CP, Nedelec L, Tutty MA, Shanafelt T. The association between perceived electronic health record usability and professional burnout among US physicians. In *Mayo Clinic Proceedings*. 2020 Mar 1 (Vol. 95, No. 3, pp. 476–487). Elsevier.
27. Rienzi L, Fauser B. Future challenges for clinical embryologists. *Reprod Biomed Online*. 2021;43(6):973–5.
28. Niederberger C, Pellicer A, Cohen J, Gardner DK, Palermo GD, O'Neill CL, Chow S, Rosenwaks Z, Cobo A, Swain JE, Schoolcraft WB. Forty years of IVF. *Fertil Steril*. 2018;110(2): 185–324.
29. Meseguer M, Kruhne U, Laursen S. Full in vitro fertilization laboratory mechanization: Toward robotic assisted reproduction? *Fertil Steril*. 2012;97(6):1277–86.
30. Gardner DK. The way to improve ART outcomes is to introduce more technologies in the laboratory. *Reprod Biomed Online*. 2022;44(3):389–92.
31. Swain J, VerMilyea MT, Meseguer M, Ezcurra D. AI in the treatment of fertility: Key considerations. *J Assist Reprod Genet*. 2020;37(11):2817–24.
32. Go KJ. 'By the work, one knows the workman': The practice and profession of the embryologist and its translation to quality in the embryology laboratory. *Reprod Biomed Online*. 2015;31(4): 449–58.
33. Garcia EC, Garcia EC, Kundu I, Kelly MA, Guenther GA, Skillman MPASM, Frogner BK, PhD (2021) The Clinical Laboratory Workforce: Understanding the Challenges to Meeting Current and Future Needs. American Society for Clinical Pathology and Center for Health Workforce Studies, University of Washington.
34. Ground-breaking Study by ASCP and University of Washington Proposes Innovative Strategies to Address Laboratory Workforce Shortage. 2021. <https://www.ascp.org/content/news-archive/news-detail/2021/05/17/groundbreaking-study-by-ascp-and-university-of-washington-proposes-innovative-strategies-to-address-laboratory-workforce-shortage#>.
35. Klonoff DC, Kerr D. Digital diabetes communication: there's an app for that. *J Diabetes Sci Technol*. 2016;10(5):1003–5.
36. Curchoe CL. Smartphone applications for reproduction: From rigorously validated and clinically relevant to potentially harmful. *EMJ Repro Health*. 2020;6(1):85–91. <https://doi.org/10.33590/emjreprohealth/20-00033>.
37. Maitra A, Tripathi S, Shankar A. Telemedicine: An application of cloud computing. In *Recent Trends in Communication and Electronics*. Boca Raton, FL: CRC Press, pp. 456–459, 2021.
38. The Society For Assisted Reproductive Technology. <https://apps.apple.com/us/app/sart-mobile/id1370121034>.
39. Agarwal A, Selvam MK, Sharma R, Master K, Sharma A, Gupta S, Henkel R. Home sperm testing device versus laboratory sperm quality analyzer: Comparison of motile sperm concentration. *Fertil Steril*. 2018;110(7):1277–84.
40. HFEA. Code of Practice (2018) T24, Annex 13, 26. Equipment and materials. Pg 165, 2018.
41. Alikani M. Cryostorage of human gametes and embryos: A reckoning. *Reprod Biomed Online*. 2018;37(1):1–3.
42. Jury Finds Tank Maker Responsible for Lost Eggs and Embryos, Awards \$15 Million. <https://www.courthousenews.com/jury-finds-tank-maker-responsible-for-lost-eggs-and-embryos-awards-15-million/>.
43. US Litigation relating to: No. 3:20-cv-05047 (A.J. and N.J.) No. 3:20-cv-04978 (L.E. and L.F.) No. 3:20-cv-05030 (M.C. and M.D.) No. 3:20-cv-04996 (O.E.) No. 3:20-cv-05041 (Y.C. and Y.D.). <https://casetext.com/case/in-re-pac-fertility-ctr-litig-9>.
44. Pomeroy KO, Marcon M. Reproductive tissue storage: Quality control and Management/Inventory software. *Semin Reprod Med*. 2018;36(5):280–288. doi: [10.1055/s-0038-1676851](https://doi.org/10.1055/s-0038-1676851).
45. Palmer GA, Parker PA, Dawson-Smith KJ, Igbokwe NN. Improving sample identification and reducing risks during cryostorage of vitrified gametes and embryos using radio frequency identification (RFID) tags that operate while samples are immersed in liquid nitrogen. SLTB 2019 – Seville, 04 October 2019.
46. Logsdon DM, Grimm CK, Schoolcraft WB, McCormick S, Schlenker T, Swain JE, Krisher RL, Yuan Y, Collins MG. Evaluation of the TMRW vapor phase cryostorage platform using reproductive specimens and in vitro extended human embryo culture. *Fertil Steril*. 2021;2(3):268–77.
47. Mogal AK. Wireless mobile communication-a study of 3G technology. *Int J Adv Network Appl*. 2012;3(5):1.
48. Rothman BS, Gupta RK, McEvoy MD. Mobile Technology in the perioperative arena: Rapid evolution and future disruption. *Anesth Analg*. 2017;124(3):807–18.
49. US Department of Health and Human Services and US Office for Civil Rights, 1996; Health Insurance Portability and Accountability Act of 1996. <https://www.hhs.gov/hipaa/for-professionals/security/laws-regulations/index.html>.
50. Officer of Privacy Commissioner of Canada: Personal Information Protection and Electronic Documents Act, 2019. <https://www.priv.gc.ca/en/privacy-topics/privacy-laws-in-canada/the-personal-information-protection-and-electronic-documents-act-pipEDA/>.
51. Australian Government. Office of the Australian Information Commissioner. The Privacy Act, Australia, 1988. <https://www.oaic.gov.au/privacy/the-privacy-act>.
52. The European Parliament and the Council of the European Union. Directive 95/46/EC (General Data Protection Regulation), 2016. <https://eur-lex.europa.eu/eli/reg/2016/679/oj>.
53. Govender I, Watson BW, Amra J. Global virus lockdown and cybercrime rate trends: A routine activity approach. *J Phy: Conf Ser*. 2021;1828(1):012107.
54. BBC Hack leaves fertility clinic medical data at risk. 2022 <https://www.bbc.co.uk/news/technology-59156683>.
55. Wood CC, Banks WW Jr. Human error: An overlooked but significant information security problem. *Comput Secur*. 1993;12(1): 51–60.

56. Kumar T, Ramani V, Ahmad I, Braeken A, Harjula E, Ylianttila M. Blockchain utilization in healthcare: Key requirements and challenges. In 2018 IEEE 20th International Conference on e-Health Networking, Applications and Services (Healthcom). 2018 Sep 17 (pp. 1–7). IEEE.
57. Hickman CF, Alshubbar H, Chambost J, Jacques C, Pena CA, Drakeley A, Freour T. Data sharing: Using blockchain and decentralized data technologies to unlock the potential of artificial intelligence: What can assisted reproduction learn from other areas of medicine? *Fertil Steril*. 2020;114(5):927–33.
58. Cristian F. Understanding fault-tolerant distributed systems. *Commun ACM*. 1991 Feb;34(2):56–78.
59. Huff MA (2017). MEMS. In Internet of Things and Data Analytics Handbook, H. Geng (Ed.). Wiley Online Library, NJ. <https://doi.org/10.1002/9781119173601.ch9>.
60. Sultan N. Making use of cloud computing for healthcare provision: Opportunities and challenges. *Int J Inform Manag*. 2014;34(2):177–84.
61. Russell CL. 5 g wireless telecommunications expansion: Public health and environmental implications. *Environ Res*. 2018;165: 484–95.
62. Cisco (2020) Number of IoT Devices Expected to Reach 24.1 Bn in 2030: Report <https://transforma insights.com/news/iot-market-24-billion-usd15-trillion-revenue-2030>.
63. Jie Y, Pei JY, Jun L, Yun G, Wei X. Smart home system based on IoT technologies. In 2013 International Conference on Computational and Information Sciences. 2013 Jun 21 (pp. 1789–1791). IEEE.
64. Pavithra D, Balakrishnan R. IoT based monitoring and control system for home automation. In 2015 Global Conference on Communication Technologies (GCCT). 2015 Apr 23 (pp. 169–173). IEEE.
65. Dang LM, Piran M, Han D, Min K, Moon H. A survey on internet of things and cloud computing for healthcare. *Electronics*. 2019;8(7):768.
66. Sargunam B, Anusha S IoT based mobile medical application for smart insulin regulation. In 2019 IEEE International Conference on Electrical, Computer and Communication Technologies (ICECCT). 2019 Feb 20 (pp. 1–5). IEEE.
67. Chapman KR, Barnes NC, Greening AP, Jones PW, Pedersen S. Single maintenance and reliever therapy (SMART) of asthma: A critical appraisal. *Thorax*. 2010;65(8):747–52.
68. Lakshminarayana R, Wang D, Burn D, Chaudhuri K, Galtrey C, Guzman NV, Hellman B, James B, Pal S, Stamford J, Steiger M. Using a smartphone-based self-management platform to support medication adherence and clinical consultation in Parkinson's disease. *NPJ Parkinson's Disease*. 2017 Nov 13;3(1):1–9.
69. Yang G, Xie L, Mäntysalo M, Zhou X, Pang Z, Da Xu L, Kao-Walter S, Chen Q, Zheng LR. A health-IoT platform based on the integration of intelligent packaging, unobtrusive bio-sensor, and intelligent medicine box. *IEEE Transact Industr Informat*. 2014 Feb 24;10(4):2180–91.
70. Qiu H, Wang X, Xie F A survey on smart wearables in the application of fitness. In 2017 IEEE 15th Intl Conf on Dependable, Autonomic and Secure Computing, 15th Intl Conf on Pervasive Intelligence and Computing, 3rd International Conference on Big Data Intelligence and Computing and Cyber Science and Technology Congress (DASC/PiCom/DataCom/CyberSciTech). 2017 Nov 6 (pp. 303–307). IEEE.
71. Horcher AM. A tap on the wrist: Security usability for wearables. In Proceedings of the 2nd workshop on inclusive privacy and security. 2015 Jul 22.
72. Amazon Technologies Inc. (2018) Wristband haptic feedback system, Patent No. US 9,881,273 B2.
73. Sra M, Panda K, Rath S. A web of IOT sensors to automate quality control in an IVF embryology lab. *J Int Soc Telemed eHealth*. 2017;5(GKR):e55.
74. Kanawday A, Sane A. Machine learning for predictive maintenance of industrial machines using IoT sensor data. In 2017 8th IEEE International Conference on Software Engineering and Service Science (ICSESS). 2017 Nov 24 (pp. 87–90). IEEE.
75. Khanagha S, Ansari S, Paroutis S, Oviedo L. Mutualism and the dynamics of new platform creation: A study of cisco and fog computing. *Strateg Manag J*. 2022;43(3):476–506.
76. Abdalah AN, Mohamed A, Hefny HA. Proposed authentication protocol for IoT using blockchain and fog nodes. *IJACSA*. 2020;11(4). <http://dx.doi.org/10.14569/IJACSA.2020.0110491>.
77. Novo S, Barrios L, Santalo J, Gómez-Martínez R, Duch M, Esteve J, Plaza JA, Nogués C, Ibáñez E. A novel embryo identification system by direct tagging of mouse embryos using silicon-based bar-codes. *Human Reprod*. 2011;26(1):96–105.
78. Weiser M. Some computer science issues in ubiquitous computing. *Commun ACM*. 1993;36(7):75–84.

5

THE ASSISTED REPRODUCTION TECHNOLOGY LABORATORY

Current Standards

Cecilia Sjöblom

Introduction

Quality assurance (QA), quality control (QC), and accreditation are concepts that seem to touch on a wide range of functions in our society. QC systems and standardization are especially needed in units for assisted reproduction technology (ART) to ensure the reproducibility of all methods and that all members of staff are competent to perform their duties. The necessity of a QC system becomes even clearer when considering the possible risks of ART.

Over the years that ARTs have been practised, extensive knowledge has been gained on how to run an ART laboratory and what methods to use to achieve ultimate success. Facing the future, we encounter other variables such as the safety and efficiency of the laboratory, and quality and standardization become key features. Professional, national, and international guidelines on how ART should be performed have been established over the years, and many countries have legislation concerning how ART should be practised [1]. Among others, England, Australia, and the United States have instituted a system whereby the ART clinics have to be licensed to practise these techniques and the clinic and the laboratory are audited by a third-party authority in order to ensure correct practice [2–5]. However, with the increased knowledge of the importance of implementing quality systems, most clinics choose to conform to any of a range of available standards.

This chapter first provides an overview of the most common laboratory standards together with some regional/national guidelines and regulation. Then, it provides a simple “how-to” guide for laboratories seeking to conform to internationally recognized standardization. Then, most importantly, it goes beyond the standards to establish some key determinants of success, which are interdependent for maintaining high-quality standards, safety, and improved results in the *in vitro* fertilization (IVF) laboratory.

Standards

International standards and regulatory frameworks

International Organization for Standardization (ISO) 9001, with its current version 9001:2015 Quality Management Systems—Requirements [6], is the most widely used standard in ART clinics and involves the quality system of the whole organization. This standard covers the need for quality management and the provision of resources (both personnel and equipment), and a substantial section involves customer satisfaction and how to improve services. A more detailed overview of ISO 9001:2015 is presented in Chapter 32 [7].

ISO 17025:2012, specifying general requirements for the competence of testing and calibration laboratories [8], is the main international standard for laboratory accreditation. It is based on the European norm (EN) 45001 [9] and was originally modelled on the corresponding ISO/International Electrotechnical

Commission (IEC) guide [10]. The scope of this standard is specialized and is aimed towards assurance of methods and includes both the quality system and the technical part of the activities such as validations of methods, QA, QC, and calibration of equipment. In 1997, Fertility Centre Scandinavia became the first IVF laboratory to be accredited according to this international standard [11].

With an increase in laboratory accreditation, it was evident that ISO 17025, aiming to standardize testing and calibration laboratories, could not fully accommodate and cover the complexities of a medical testing laboratory. ISO 15189, on medical laboratories, particular requirements for quality, and competence, was issued to aid the accreditation of methods used in medical testing. It was first issued in 2003, with the current fourth edition issued in December 2022 [12]. It is used for the accreditation of medical laboratories and brings together the quality system requirements of ISO 9001 and the competency requirements of ISO 17025 and addresses the specific needs of medical laboratories.

Most medical laboratories in Europe and Australia are accredited according to ISO 15189. There are differences between the two laboratory standards, with ISO 15189 focusing on patient outcome without downgrading the need for accuracy, and it emphasizes not only the quality of the measurement but also the total service provided by a medical lab. The language and terms are familiar to the medical profession, and it highlights important features of pre- and post-investigational issues while also noting ethics and the information needs of the medical laboratory. ISO 15189:2022 is risk-based and addresses the need for equivalency of quality management systems and competency requirements between laboratories. The need for this becomes more obvious at a time when potential and actual patients are increasingly mobile—the systems to collect medical data on these patients must be standardized independently from their location.

IVF laboratories located in the European Union (EU) are required to adhere to the Directive on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells, usually called the European Union Tissue and Cells Directive (EUTCD) and its guide/supporting documents [13–17]. The European Society of Human Reproduction and Embryology (ESHRE) has issued a position paper on the EUTCD [18], and it is important to underline that regardless of ESHRE’s recommendations, each EU country interprets the Directive differently. However, one part of the EUTCD is very clear: the demand for a quality system. The Directive states that “Tissue establishments shall take all necessary measures to ensure that the quality system includes at least the following documentation: standard operating procedures (SOPs), guidelines training and reference manuals.” Certainly, by achieving accreditation to ISO 15189, this demand will be fulfilled along with several other demands of the Directive. The EUTCD is currently being updated, and

in July 2022, the European Commission published a “proposal for a regulation of the European parliament and of the council on standards of quality and safety for substances of human origin intended for human application and repealing Directives 2002/98/EC and 2004/23/EC” [19]. It will take into account technological, epidemiological, and clinical developments in ART [20]. While welcoming the initiative and being largely supportive of the proposals, ESHRE is preparing a position statement on the proposals [21].

Joint Commission International (JCI) is a non-profit organization with the main focus on improving patient safety credit, and they certify hospitals and healthcare organizations worldwide. JCI has a range of standards including Accreditation Standards for Clinical Laboratories [22]. The World Health Organization (WHO) in collaboration with JCI has developed a core program for patient safety solutions. It brings attention to patient safety and practices, which can help reduce the risks involved with medical procedures. The most recent advice builds on “nine patient safety solutions” including patient identification and recommends actions in four basic categories: (i) risk management and quality management systems; (ii) policies, protocols, and systems; (iii) staff training and competence; and (iv) patient involvement [23].

The Clinical and Laboratory Standards Institute (CLSI) is another global not-for-profit standards development organization, and while mostly applicable to the United States, the CLSI standards are of great help for improving laboratory quality and safety [24].

In the United States, the College of American Pathologists (CAP) in collaboration with the American Society for Reproductive Medicine (ASRM) has developed a standard that meets requirements of reproductive laboratories. The Reproductive Accreditation Program promotes the quality and safety of laboratories and is recognized by the Society for Assisted Reproductive Technologies (SART) to meet membership requirements for IVF facilities [25].

Other standards that might be less suitable for the IVF laboratory are the Good Manufacturing Practice/Good Laboratory Practice (GMP/GLP) guides. These standards apply to research laboratories and the pharmaceutical production industry. They include demands on the laboratory facilities that will be difficult to meet with the limited resources that many IVF clinics have [26, 27].

In addition to these quality system-driven standards, there are many IVF-specific standards and guidelines including WHO laboratory manuals for the examination and processing of human sperm [28] and the Alpha/ESHRE consensus papers on embryo assessment [29] and ART laboratory performance indicators [30]. The Alpha consensus group has published a consensus for cryopreservation establishing key performance indicators (KPIs) and benchmarks for both slow freezing and vitrification [31].

National and regional standards

While the ISO standards cover the fundamental needs for quality systems in the IVF laboratory, many regions and countries have specific guidelines, laws, and regulations. It is important to note that while some of these regulatory frameworks are standards and others are license requirements or law, when it comes to inspections and audits, the laboratory is expected to conform.

Europe

With the EUTCD in place, all IVF laboratories handling gametes and embryos are required to have a quality system and to fulfil the demands of the Directive and the national interpretation of it.

This has led to most of the IVF laboratories in the EU holding or working towards formal accreditation to ISO 15189 or ISO 17025. As described earlier in this chapter, ESHRE has published revised guidelines for good practice in IVF laboratories, providing an easy-to-navigate guide to support laboratory specialists and also to fulfil some of the demands of the Directive [32]. In the UK, where all IVF clinics are required to be licensed by the Human Fertilisation and Embryology Authority (HFEA) [33], there are further guidelines regulating the IVF laboratory as detailed in the HFEA Code of Practice (HFEA CoP) [34]. Specifically, the CoP contains demands for risk management, sample identification, and embryology staffing as described later in this chapter. Following the departure of the UK from the EU, new HFEA EU exit legislation was introduced [35]. Following this, the HFEA has made changes to the standard licensing conditions reflecting some of the core requirements of the EUTCD with regards to laboratory air quality, traceability, medical devices, and importing of tissue [36].

Australia and New Zealand

In Australia, the Reproductive Technology Accreditation Committee (RTAC) undertakes the licensing of IVF clinics. While the RTAC CoP [5] is far less comprehensive than its UK counterpart, it contains critical criteria with a focus on risk management, staffing, and sample identification as well as further guidelines covering the requirement of a quality management system. In addition to the code, Fertility Society Australia issues technical bulletins, which act as educational communication to all units and certifying bodies, offering advice and guidance. It is not enforceable [37]. In New Zealand, RTAC licensing is optional, but most clinics hold an RTAC license. While the majority of IVF clinics hold an ISO 9001 certification, most are also accredited by National Association of Testing Authorities (NATA) to ISO 15189 for some of the crucial methods such as semen analysis. However, very few laboratories hold ISO 15189 accreditation for the overall IVF laboratory processes.

Asia

At the time of publication, there were very few IVF laboratories in Asia accredited according to ISO 15189 or similar standards and the laboratory accreditation was not widespread. However, there is an increased interest and need for standardization. Many private IVF centres throughout the region have ISO 9001 certification.

Memorial Hospital in Istanbul, Turkey, was the first IVF laboratory in the region to achieve ISO 15189 accreditation (acknowledging that Turkey is a transcontinental country at the junction of Europe and Asia).

With the introduction of strict regulations of ART in China, it has become increasingly challenging to obtain approval to operate an ART centre. The Ministry of Health issued the first series of regulations on ART in 2001 which remain in current [38]. These regulations have detailed requirements with respect to facilities, staff, equipment, clinic management, QA/QC, indication and contraindication of IVF, intracytoplasmic sperm injection (ICSI), egg donation, and preimplantation genetic testing (PGT), among others, along with the ethical aspects of various issues. In addition, since 2006, the department requires control inspections of ART providers to be done every two years and that there is provision for accredited training of ART specialists. The Chinese Society of Reproductive Medicine of the Chinese Medical Association is actively engaged in detailed

ART treatment guideline establishment and implementation. In 2015, the Health Commission issued the Guidelines for the Configuration Planning of ART (2015 edition) to guide the scientific planning of all provinces (autonomous regions and municipalities) for the development of ART from 2015 to 2020 with a new edition published in 2021 [39]. It is proposed in the Guidelines that a Plan for the Application of ART (2021–2025) be formulated by all regions, with the medical institutions carrying out ART in their respective provinces (autonomous regions and municipalities). It is clarified in the Guidelines that, when formulating this Application Plan, all regions shall pay more attention to guaranteeing social public welfare, optimizing the efficiency of resource supply, and improving the service quality. The compilation principles include quality and safety priority, classified guidance and management, reasonable planning and layout, and stable and orderly development [39].

ART in India is governed by the recently gazetted Assisted Reproductive Technology (Regulation) ACT (December 2021) and a law on surrogacy (January 2022) [40]. The Indian Council of Medical Research (ICMR) has issued National Guidelines for Accreditation, Supervision, and Regulation of ART Clinics (not legislated) [41]. The Guidelines cover issues such as staff qualifications and laboratory procedures, but neither the ACT nor the ICMR Guidelines have a formal demand for quality systems. In November 2021, the Indian Society for Assisted Reproduction (ISAR) published Consensus Guidelines on Safety and Ethical Practices in In vitro Fertilization Clinics with the aim of helping embryology laboratories across India to standardize their practices and improve outcomes [42]. The guidelines cover a wide range of topic but importantly highlight the need for QC, staffing training and qualifications, safety/risk management, and ID and traceability. ISO certification is not widespread for individual IVF clinics, but larger hospitals that have IVF departments are commonly ISO certified.

In Japan, IVF services have historically been charged out of pocket with a Grant system covering some of the costs; however, this was changed at the end of March 2021. From April 2022, IVF and related services are covered by the national insurance system. Leading up to the introduction of IVF in the national insurance system, the Japan Society of Obstetrics and Gynecology (JSOG) developed a clinical guideline which not only forms the basis for what to include and what not to include in the insurance system but also provides some guidance on the evidence level for treatment practices and technologies. Even though JSOG has issued other guidelines covering IVF, there is no single guideline that comprehensively covers laboratory practices and the need for quality systems. As a result, some clinics have created their own umbrella organizations for implementing common quality practices within IVF called the Japanese Institution for Standardizing Assisted Reproductive Technology (JIS-ART) [43].

In Singapore, the Ministry of Health has introduced stringent licensing requirements for assisted reproduction services in private hospitals and clinics. It covers demands for QC, facilities, embryology training, and sample identification [44]. All IVF providers in Singapore are accredited according to the international version of the RTAC CoP [45].

Middle East

ART in many Muslim countries is covered by a number of fatwas (religious opinion concerning an Islamic law issued by an Islamic scholar) [46]. The first fatwa relating to ART was issued in 1980 by His Excellency Gad El Hak Ali Gad El Hak, the Grand Sheikh

of Egypt's Al-Azhar Mosque. The core requirement is that the couple is married, and the use of donor sperm or oocytes is prohibited [47].

Apart from the fatwas, there are very few regulations and standards for IVF laboratories in the Middle East and few laboratories are formally accredited to international standards, but many larger hospitals hold JCI and ISO accreditations.

Saudi Arabia has a comprehensive fatwa containing demands for documented SOPs, safeguarding of sample ID, and prevention of mix-ups, among others. The Ministry of Health has started setting standards, and some centres have had audits by the authorities [46].

Centrally located, luxurious, and tourist friendly, the United Arab Emirates (UAE) attracts IVF patients from all over the Middle East and Europe. The UAE has stringent laws regulating IVF and requires that all centres are licensed by the Department of Health (DOH) with all laboratories being regularly audited. The Federal Law No. (07) Concerning Medically Assisted reproduction was updated in 2019 [48], with the DOH Standard for Assisted Reproductive Technology Services and Treatment issued in 2022 to direct the implementation of the new law. The Standard demands that laboratories must obtain accreditation either from the CAP Reproduction [25] or ISO [12] within 6–12 months of establishment. Existing laboratories are required to meet these accreditation demands by February 2023 [49]. The regulations include demands on the embryology staff having master's degree and PhD and stipulates requirements for ongoing training, laboratory facilities, documented protocols and procedures, and QC.

A recent review by Dr Chokri Kooli provides a great overview of ART laws and regulations in Muslim countries in the Middle East and beyond [50].

Latin America

Registro Latinoamericano de Reproducción Asistida (Red LARA) covers most of the Latin American clinics. Although membership in the organization is voluntary, 70%–80% of clinics participate in the data collection, accreditation, and continuous professional development training programs. The accreditation includes external audits and follows the Standard Rules for the Accreditation of the ART centre and its laboratories of embryology and andrology [51] involving, among others, QC, KPIs, staff requirements, equipment, and materials.

Each country has individual health authority regulations that must be adhered to when establishing a new IVF centre/laboratory, with many taking advice from colleagues in the United States. ASRM is offering accreditation of laboratories outside the United States; however, more commonly, embryologists establish laboratory facilities and processes according to their knowledge considering their financial possibilities and what they learn from clinics from around the globe.

Each country also has local embryology societies. In Argentina, Asociación para el Estudio de la Biología de la Reproducción (ASEBIR) has a special interest group on laboratory quality for the members of the society. ASEBIR has developed a calculator named Cassandra that calculates the staff number and time required for performing all embryology laboratory activities in order to assure safe staffing levels [52].

North America

In 2004, the Canadian federal government passed the Assisted Human Reproduction (AHR) Act with Health Canada holding

the responsibility for federal functions related to assisted human reproduction [53, 54]. In 2016, Health Canada announced that they intended to make sections of the Act enforceable, notably section 10 of the AHR Act, on the Safety of Sperm and Ova Regulations which came into force in 2020 [54]. The Guidance Document Safety of Sperm and Ova Regulations covers a range of areas such as gamete and embryo handling, quality management, staff requirements, and facilities [55]. Additionally, The Canadian Fertility and Andrology Society (CFAS) has published a number of clinical practice guidelines and is working towards comprehensive professional standards concerning the laboratory activities involved in IVF prepared by its ART Lab Special Interest Group, along with training and competency requirements that include the continuing professional development of all ART laboratory scientists [56].

In the United States, the practice committees of the ASRM and the Society for Reproductive Biologists and Technologists (SRBT) have recently issued updated comprehensive guidelines for human embryology, andrology, and endocrinology laboratories [57, 58]. This publication provides guidance on embryology laboratory staff minimum requirements for education, training, continuing education, and experience. It further gives a very clear overview of US laboratory certification and accreditation requirements, regulatory obligations with regards to the US Federal Food and Drug Administration (FDA), and implementation of Quality Management Systems [58].

How to achieve laboratory accreditation

It is important to underline that in no way are all the quality standards independent of each other. The new ISO 15189:2022 is modelled on ISO 17025:2017, with the major difference being the medical laboratory terminology used in ISO 15189. In turn, both are aligned to ISO 9001:2015, and as such, the new ISO 15189 standard is risk based with the patient in focus prompting laboratories to take the risk to patients into account in both processes and quality management. ISO 15189:2022 also allows for more flexibility than previous standards with formulations like “where applicable” following “shall” stipulations. This allows the laboratory to take their own circumstances into account when applying the standard. The quality system requirements of both standards (17025 and 15189) are based on ISO 9001. As a result of this, laboratories within ISO 9001-certified clinics seeking accreditation will have major parts of the system requirements of the two laboratory standards already in place. It could be recommended that the first step towards accreditation is to get the clinic certified to ISO 9001; further details on this subject are found in Chapter 32. The requirements discussed throughout the continuation of this part of the chapter will be for laboratory accreditation to ISO 15189 or ISO 17025 on top of (over and above) what is already required for certification to ISO 9001. For example, scope, organization, and document control are found in all the standards, and many of the demands are the same, but the requirements further discussed in this chapter will be what ISO 15189 has (hereinafter referred to as the standard) in addition to what has already been implemented through ISO 9001 certification. Correlation tables for ISO 9001, 17025, and 15189 can be found in the standards themselves.

Getting Started

The first step towards an accreditation is to make sure that everyone in the organization wants to achieve the same goal. The full understanding of how everyone benefits from an

accreditation will make the process easier. A good way to ensure this is to have staff meetings throughout the process and involve all staff from the very beginning. The most frequent mistake organizations make when trying to implement a QC system is not to involve everyone. Divide the project into smaller sections and give out personal responsibilities enabling all staff to be included in the preparation work. This will also make the implementation easier.

A good way to make sure that all demands in the standard are covered is to make up a table of contents using the ISO 15189:2022 standard table of contents as a template. An assessment can then be made of what needs to be added to the quality manual and other documentation. It is important to note that while the standards have demands for management structure, internal audit, or document control, the laboratory standards have some more specified demands not found in ISO 9001, and these need to be added to the specific procedures.

Methods and SOPs

Examination processes (ISO 15189:2022; 7.3)

The methods and processes we use in the embryology laboratory and their efficacy have a direct impact on the pregnancy results of the clinic. It is therefore hugely important that we standardize these methods and make sure that they are reproducible. In simple words, an ICSI should be done in the same way using the same disposals and equipment by all embryologists in the lab, ensuring that an ICSI done by embryologist A on a Monday is performed in exactly the same way and with the same level of skill as an ICSI done by embryologist B on a Friday. Ensuring the performance of correct methods is achieved through several steps. First, we need to make sure that the processes and methods we use are correct and up to date with the latest developments in ART. Hence, a clear starting point should be a literature search, together with the knowledge gained from workshops, external training, and visits to other clinics. The standard stipulates that we “shall select and use methods which have been validated for their intended use to assure the clinical accuracy.” Once the details of the methods have been agreed between the embryology team members, they need to be documented. A document describing a method or process used in a laboratory is commonly called an SOP. A good SOP should follow a set format, and the old ISO 15189:2012; 5.5.3 contains a very good guide for SOP layouts [59]. The SOP title should be followed by a short clinical description of the method. The analytic principles need to include a theoretical description of the method and review of the current literature. The SOP should outline the competence demands on embryologists performing the process. Collection and handling of gametes and embryos should include the sampling procedures and the physical environmental issues such as temperature. Remember that all variables in the SOP, such as those referring to the measurement of temperature, have to give a precise range, followed by a description of how the temperature is measured, the accuracy of the thermometer, and how often and how it is calibrated. There should be clear descriptions of how the sample is labelled and, considering the risks associated with the work in an IVF laboratory [60], the marking should be logical and clear in order to eliminate completely the risk of mixing of samples (for further details, refer to the “Sample identification, witnessing, and prevention of misidentification” section later in this chapter). The description of the procedural steps should be written in an

uncomplicated way so that they can be easily followed by any new member of staff under supervision.

All equipment used for the method should be listed with references to handling instructions and calibration protocols. Any safety routines and occupational hazards involved should be discussed and clearly known by the embryologists. References to any textbooks or publications concerning the method should be included last.

The standard demands that the procedures used should meet the requirements of the users of the laboratory service, preferably applying methods that have been published in established/authoritative textbooks, peer-reviewed texts, or journals. If in-house methods are used, these need to be appropriately validated for the intended use and fully documented by the laboratory. The standard requires that all documentation relating to the processes and all supporting documentation be readily available to personnel (7.3.1 c), preferably digitally as the use of paper should be minimized in the clean facilities (embryology laboratory).

When the SOP is written, it needs to be communicated to all members of the embryology team, and it is important to allow them to comment, give feedback, and suggest changes before the document is formally issued and implemented. The way to check that all embryologists follow the new SOP is to undertake audits, and it is suggested to audit all processes three months after the issue of the SOP. If the audit findings include discrepancies between the written SOP and the embryologists' hands-on working procedure, then either the SOP needs to be changed to reflect the actual hands-on procedure or the member of staff needs to be retrained and reminded of the importance of following the agreed SOP. No embryologists can insist on doing things "their own way" in a standardized high-quality IVF laboratory.

Once the SOPs are fully implemented and the audits show that we have achieved the required reproducibility, then we need to ask: is it working? Is the method we agreed upon successful? The standard calls this "verification" and "validation" (7.3.2), and it is the process that confirms that the techniques and methods used in the IVF laboratory are suitable to produce good embryos, viable pregnancies, and live births. All methods must be validated regularly, and the SOP should include information on how often and how validations are done. The EUCTD includes demands for validation, and in the UK, the HFEA CoP [34] requires that all processes in the IVF laboratory be validated. Some methods and techniques used in the laboratory can be difficult to validate, and it is acceptable to use retrospective analysis of fertilization, damage, and pregnancy rates to validate ICSI and IVF. Appropriate validation of new techniques can become very difficult when considering the sample size needed to prove a null hypothesis or small increase in pregnancy rates. An accurate validation of a new culture medium will need hundreds of patients in each study group. Adding to the complexity of validation practice is the fine line between validation and research, and questions are raised regarding the need for ethical approval to undertake validations [61]. However, it is highly recommended to regularly validate other practices in the lab, such as changes of osmolarity during preparation of dishes, temperature fluctuation during denudation, and temperature distribution in incubators. Validation of temperature in a culture medium in different types of dishes on all heated stages in the laboratory should confirm the appropriate range of surface temperature of the heated stage.

Handling of gametes and embryos

Pre- and post-examination process

(ISO 15189:2022; 7.2, 7.4)

The standard has specific demands on how the sample—that is, gametes and embryos—should be collected and stored, noting that the pre-examination process can influence the outcome of the intended process. The samples must be correctly and safely identified, and any laws regulating the identification of patient samples have to be considered (for further details, refer to the "Identification, witnessing, and prevention of misidentification" section later in this chapter). The sample should be accompanied by a written, standardized request of what procedure the sample should be used for. It is a common occurrence that the requests for treatment are unclear and that couples who could have had conventional IVF end up having ICSI due to poor communication. Senior embryologists with considerable experience in assessing sperm samples are more suitable to making the final decision on IVF or ICSI in conjunction with the couple on the day of treatment when the sample has been washed than the referring doctor who takes the decision on IVF or ICSI based solely on a semen analysis report. Other procedures where clear requests are crucial are frozen embryo transfer (ET) cycles to ensure that the embryo is thawed at the correct time assuring endometrium/embryo synchronization. For collection of sperm, the date and time of collection should be noted by the patient and the date and time of receipt should be recorded by the laboratory. Noting sperm sample collection time is important as an ejaculated sample needs to be processed within 40 minutes of production. Delay in processing with prolonged sperm—seminal fluid (reactive oxygen species [ROS] exposure)—can cause increased sperm DNA damage/mutational loads resulting in poor fertilization outcome and embryo development. Section of the standard 7.2.4.2 provides a guide on what information the laboratory must include. Assuring patient consent is another crucial part of the pre-examination process (12) and the post-examination process (7.2.4) specifically with regards to cryo-preserved material.

Usually, the procedures for collecting samples at pre- and post-examination are documented in the applicable laboratory SOPs for sperm processing and oocyte collection. However, it is important to include the specific demands of the standards for these procedures and the documentation of them.

Laboratory sheets and reports

Reporting and releasing results (ISO 15189:2022; 7.4)

The details from assessments of gametes and embryos we document in the laboratory on lab sheets are referred to in the standard as reports. The reporting of results should always be accurate, clear, unambiguous, and objective. This requires that the lab sheets be standardized and follow a set format. They should be filled out in a neat manner—no scribbling allowed. All entries and comments on a lab sheet should be accompanied by a date and signature. For sperm assessment, sources of errors and uncertainty of measurements should be stated and properly calculated for each method. Formal reports, such as seminal fluid analysis reports, should also be checked and signed off by the senior andrologist/embryologist before being issued.

Many laboratories have computerized databases and enter the information from the lab sheets into the database. It is important to understand that the handwritten lab sheet is considered source data and therefore needs to be archived correctly, not destroyed

after computer entry. If the laboratory wants to go paper free, it has to indeed be paper free and allow for direct data entry onto the computer without an in-between paper sheet. When considering the need for signatures and witnessing, a complete paperless IVF laboratory could be difficult to create. The standard provides a good overview of what is required with regards to reports in (12).

The embryology laboratory

Facilities and environmental conditions (ISO 15189:2022; 6.3)

A laboratory needs to ensure that the environmental conditions of the laboratory are suitable for the safe handling of gametes and embryos and do not invalidate the results or adversely affect the quality of any procedure. In simple words, this means that the IVF laboratory must be designed in such a way that the outcome of any procedure is optimal and not affected by environmental parameters. The standard further requires us to consider the safety of patients, visitors, laboratory users, and personnel.

Live birth results following IVF treatment vary from country to country and from clinic to clinic and often within a clinic from month to month. It is a general consensus that patient demographics, such as age and cause of infertility, are the main factors affecting the outcome. Considering a varying population of patients, it is of great importance that parameters in the laboratory are stable. Defining the environment and setting limits for acceptable working conditions will help with reducing variables and result in the patient being the only factor that varies. Exactly what this encompasses will always be down to interpretation and international, national, or regional regulations; however, the standard has some clear demands, and some environmental factors cannot be ignored.

General laboratory layout

The theatre for oocyte retrieval and ET should be in close vicinity to the laboratory. The laboratory layout should further ensure safe handling of gametes and embryos; small, crowded laboratories impose a significant risk for accidents, resulting in loss of gametes and embryos.

The laboratory should never double as an embryologist office. There needs to be a minimal allowance of paper in the laboratory as this can increase the amount of particles in the air. Therefore, only patient records necessary for ongoing treatment should be kept in the laboratory. Also, the laboratory is not the place for cardboard boxes as these involve a high risk of fungus infections. Furthermore, the laboratory is not a storage room for disposables; only a weekly stock of disposables should be kept inside the lab, and further storage can be managed elsewhere. The equipment held in the laboratory should be limited to only that which is absolutely necessary; again, the laboratory is not a storage room for old lab equipment.

Access rules

The standard stipulates that access to the laboratory facilities is controlled (6.3.2). The laboratory should have limited access ensured by use of locks, swipe cards, or other access controls. It should also hold documentation verifying who has access to the laboratory. There should be documented and implemented rules for what is required for access to the laboratory including demands for change of clothes and shoes, the use of hair cover and masks, and the washing of hands. Although some embryologists insist

that changing clothes and covering hair are of no importance, it is important to understand that embryology and handling of gametes and embryos are sterile processes with a need to protect the samples from microbes and contaminants. The correct degree of cleanliness is impossible to reach if the embryologists are using their own clothes or only minimal cover such as laboratory coats. Best practice is to change clothes and preferably use scrubs, which are made of low-lint, no shedding material; cotton is high lint and not advisable. Many embryologists complain that these types of scrubs are uncomfortable and that they will not use them as cotton is comfortable, but it is important to understand that we did not become embryologists to be comfortable—we need to do what is best for gametes and embryos. Further, all hair should be covered, and again some might see the cap as a fashion item that looks much better if hair is allowed outside it, but they need to be reminded to tuck in all hair before entering the laboratory. Changing into cleanroom shoes goes without saying. Best practice is to have all-white shoes with white soles in the laboratory. This makes it easy to spot any spillage on them. Also, the rack for these shoes should be designed so that the shoes are hung up with soles facing out, allowing for daily inspection of the cleanliness of the shoes. If coloured shoes are used outside the laboratory, it will be easy to spot anyone who has forgotten to change the shoes. Hands should be washed using a proper disinfectant soap before entering the laboratory. Furthermore, jewellery, nail polish, long fingernails, and perfumes should not be worn in the laboratory.

Health and safety

The laboratory is required to ensure the safety of its entire staff. This includes providing an environment that minimizes the risk of transfer of any contagious contaminants through the use of class II biosafety cabinets when handling unscreened patient materials. Further, installing low-oxygen alarms and ventilation in cryo-storage facilities (note the embryology laboratory should never double up as a cryo-storage facility).

Temperature

The optimal IVF laboratory temperature is a matter of great debate; however, it has to be defined to a limited range. Some embryologists argue that an elevated laboratory temperature benefits the embryos through reduced risk of cooling during transport from the incubator to the heated stage. However, high laboratory temperatures will provide a perfect environment for microbes and contaminants. All laboratory equipment is designed to operate at room temperature, usually defined as $23 \pm 2^\circ\text{C}$, and unless the laboratory can show process verification at a different temperature, this range will be the one demanded by the standard. A laboratory without temperature control cannot be accredited.

Light

The embryo is extremely sensitive to light exposure; however, there is a wide range of opinions on whether light in the laboratory or from microscopes will harm embryos or not. It has been very elegantly demonstrated in a large study on hamster and mouse embryos that cool fluorescent light increases the ROS production and apoptosis in blastocysts and reduces the development of live-term fetuses [62]. The embryos were handled under minimal light conditions, and the test groups were exposed to 5–30 minutes of cool white, warm white, or midday sunlight. A total of 44% of blastocysts exposed to cool white light and transferred to recipients developed to term of pregnancy (day 19), compared with 73%

in the control; 58% of blastocysts exposed to warm white light developed to term (day 19). When embryos were exposed to only one minute of sunlight, only 25% of embryos developed to term, with 35% being resorbed. In light of these findings, best practice should be to have a dim light in the laboratory and to close out any daylight.

Air quality

Another area of great debate is the demands of clean air in the laboratory, and this has also been affected by regional interpretation of the EUTCD. The standard requires that attention is paid to sterility and presence of dust, and it is highly recommended that laboratories periodically monitor the particle count and presence of volatile organic compounds (VOCs) in the air, together with microbial monitoring using contact plates for surfaces, such as replicate organism detection and counting containing Sabouraud dextrose agar (SDA; for detection of fungus) and trypticase soy agar (TSA; for detection of bacteria) and similar (TSA and SDA) settlement plates for air sampling. The plates should be exposed in key positions in the laboratory, theatre, and treatment rooms for four hours. Acceptable limits are zero colonies inside the flow hoods or handling chambers and <10 colonies outside the hoods in the laboratory.

General cleanliness

An IVF laboratory should always be clean, and the laboratory standards demand that documented frequent cleaning procedures are implemented and that cleaning is confirmed by active signatures. The use of harsh detergents is not recommended, and cleaning should be undertaken using 70% alcohol followed by sterile water or other products tested for embryology use such as Oosafe® (SparMED, Stenløse, Denmark) [63]. Steam cleaners are suitable for the cleaning of floors.

Culture medium, devices, and disposables

(ISO 15189:2022; 6.6, 6.8, 6.4)

All devices used in ART, such as culture media and consumables, will affect the outcome of the treatment. First, the laboratory needs to decide on their own requirements for culture medium, oocyte collection needles, culture dishes, and so on. This includes limits in toxicity and results from mouse embryo assays for culture media, oocyte pickup needles, or plastic ware. There is solid evidence that many of the devices and disposables we use in the embryology laboratory are indeed reprotoxic, and it is our duty to make sure that we do not use items that will expose the embryos to stress [64]. It is important to consider any national, regional, or local regulation that applies. EUTCD stipulates that all devices that come into contact with cells, gametes, or embryos need to be tested according to the EU devices directives [65, 66] and be Conformité Européenne (CE) marked. The laboratory also must define requirements for the safe transport of devices from supplier to the laboratory and how they will be inspected when they arrive to ensure they meet the limits specified. For example, there has to be a system to ensure that the box containing the culture medium is still cold when it arrives. This can easily be done by inserting a temperature probe into the box upon arrival, or requesting that the medium provider pack a temperature data logger with the medium, which you can attach to your computer when the medium arrives and ascertain that the temperature inside has been constant and correct throughout the transport. Moreover, consumables then have to be verified before taken

into use. Some laboratories choose to culture excess embryos or undertake sperm survival assays in new batches of culture medium; however, this type of verification is not demanded by the standard, and it could be argued if it is really necessary. If all the devices conform to the EU devices regulation, they should already have been stringently tested. ISO 15189 only demands that the laboratory actively checks the test reports issued by the manufacturer and confirms that the reports comply with their own limits for use.

When the devices are accepted for use, it is crucial that they are stored correctly to ensure their continued suitability for use. The laboratory must safeguard correct storage by defining the exact storage environment. Limits for temperature in refrigerators and freezers are crucial, and culture medium should be stored in a pharmaceutical refrigerator that guarantees a constant temperature throughout, whereas a normal kitchen refrigerator is not acceptable [67]. The environment in general storage rooms is also important as plastic ware stored at high temperatures will not be suitable for use.

All purchased supplies, reagents, and consumables should be included in the laboratory inventory. Information in the inventory shall include lot number (batch number), date of reception, and date taken into use. The inventory for equipment should include unique identification, date of arrival, date placed in service, last calibration or service, and periodicity of service and calibration. The laboratory is required to keep a list of approved suppliers and to critically evaluate all suppliers on an annual basis.

The batch or lot number of any device that comes into contact with a given patient's gametes or embryos needs to be recorded on that individual patient's records.

It is not appropriate to have a list of batches currently used in the lab and to draw conclusions from this using the date and guesswork of what device was used for what patient.

It is of great advantage to have a computerized case file system whereby each cycle has a batch record page attached. This page includes a full list of culture media and laboratory ware and the batches in use, and with a simple mouse click, it marks what materials were used in every step of the cycle, from culture media down to pipette tips.

Equipment

(ISO 15189:2022; 6.4)

A laboratory should have all the equipment needed to ensure provision of the best service. The standards require a documented program for preventive maintenance, and it is the responsibility of the laboratory manager to regularly monitor and ensure appropriate service, calibration, and function of all equipment. All equipment used in an accredited laboratory has to be clearly labelled with a unique identifier, date of last calibration or service, and date or expiration criteria as to when recalibration/service is due. Together with this, all equipment used should be included in an equipment record containing information listed in ISO 15189:2022; 6.4.7. There should be clearly documented processes for the validation of equipment function before it is taken into use (6.4.3). The standard of equipment used in IVF laboratories is generally very high, but even the best equipment can fail and not function optimally if it is not appropriately maintained. All embryologists should have solid knowledge of how to operate all equipment, and there should be written implemented procedures in place for action taken if there happens to be an equipment failure. Crucial equipment such as incubators should always be

connected to auto-dialers enabling staff to promptly respond to any faults out of hours.

Equipment should be verified by test runs; for example, before a new centrifuge is taken into use in the laboratory, a series of mock sperm preparations have to be undertaken and documented.

Monitoring and traceability

(ISO 15189:2022; 6.3, 6.4, 6.6, 7.3)

[Chapter 2](#) presents a detailed report of the monitoring of equipment and laboratory parameters and the traceability of reference equipment [68].

Monitoring of KPIs

Most clinics that have a quality system in place monitor KPIs. Similar to the monitoring of laboratory environmental parameters, each clinic has to agree on documented limits of performance. Usually, when monitoring parameters such as live birth, clinical pregnancy, and fertilization, there is no upper limit; however, a lower limit is necessary, along with documented plans for immediate action whenever a KPI falls under the agreed limit.

The KPIs that are essential for monitoring in connection with the laboratory include, but are not limited to, fertilization rates for IVF and ICSI, damage rates for ICSI, survival of embryos after thawing, and pregnancy results from ET. Benchmarking and KPI monitoring are hotly debated topics, and it must be underlined that trying to benchmark against a different laboratory's KPIs is a futile exercise, as laboratory performance is affected by factors such as patient selection, among other things. The best benchmarking for KPIs is done against an in-house-determined "gold standard." This is a subsection of good-prognosis patients, and the indicators for this group should be very much constant. For example, a drop in the overall KPI for fertilization with no drop in the corresponding "gold standard" indicates that the issue is related to the material coming into the laboratory. However, a drop in the KPI for the "gold standard" definitely suggests that there might be a problem with performance.

KPIs should be monitored for the whole laboratory and for each embryologist and doctor. It is important to underline the importance of confidentiality when monitoring individual performance, considering the need for the training of any embryologist falling under the given limit, but not ignoring the stress and decrease in self-confidence this can lead to. All members of staff need to understand that the monitoring is not a way of punishing people but rather to ensure that all embryologists perform to the same high standard, minimizing variables. Another important outcome of individual performance monitoring is to identify persons with exceptionally high results so that others can learn more and thereby increase the overall success.

The Alpha/ESHRE consensus group has published a detailed guide of ART laboratory performance indicators with clear examples and explanations [30]. Similarly, the ESHRE Clinic PI working group has published performance indicators for clinical practice in ART [69].

Quality assurance

Ensuring the validity of results

(ISO 15189:2022; 7.3.7)

QA makes sure that you are doing the right thing in the right way, and QC makes sure that what you have done is what you

expected. In short, QA is process-oriented and QC is product-oriented. When discussing QA/QC, it is easy to get confused; however, the terminology is not important—what is important is that the laboratory has control mechanisms in place to ensure that they perform according to the SOPs and to the highest standard. (12) and (12) demand that the laboratory has both internal QC (IQC) and external QA (EQA) in place for monitoring of the validity of the methods used. This includes the demand of internal and external controls and inter/intra-laboratory comparisons and validations. The laboratory is required to determine the uncertainty of results. This can be difficult with a subjective parameter such as embryo scoring; however, it can easily be done for the assessment of sperm. Through assessment of a series of sperm samples by all laboratory staff involved in the preparation of sperm, a coefficient of variance can be calculated, usually resulting in a 10%–15% variance.

The standard also demands that all embryologists/andrologists assess sperm samples and photos or movies of embryos on a regular basis, usually at least every three months. It is the responsibility of the laboratory manager to document the results from these comparisons, calculate variations, and address any deviance. To collect samples and photos and arrange these types of intra-laboratory comparisons takes time, and, over and above this, the standards also demand that the laboratory participates in inter-laboratory comparisons. A laboratory can share photos of embryos and samples of sperm with other centres and set up an inter-laboratory comparison scheme, although the standard clearly states that self-developed programs like this should not be used when organized external schemes are available. In the UK, most laboratories participate in the UK National External Quality Assessment Service (UK NEQAS) andrology and embryology morphology scheme, which uses online resources, DVDs, and/or formalin-fixed samples for assessment [70]. UK NEQAS collaborates with Swiss software developing company Gamete Expert and use their platform for the EQA program [71].

A web-based inter-laboratory comparison scheme is run by Dr. James Stanger and includes schemes for the assessment of all stages of human preimplantation embryos, sperm morphology and concentration, and ultrasound measurement of follicles (www.fertaid.com). The scheme provides monthly assessments of embryos and sperm and allows the laboratory manager to use the information for intra-laboratory comparison. As each of the different schemes has some 200–300 participants around the world, the intra-laboratory comparison scheme provides a solid reference for the laboratory management to implement corrective actions when deviations are found [72]. All these aforementioned EQAs are in substantial agreement with the ISO/IEC 17043:2010 Conformity assessment—General requirements for proficiency testing, which is a requirement by the standard [73].

Patient contact

Advisory services (15189:2022; 5.3.3)

In most IVF clinics, the embryologists have no or very little contact with the patient and also very little input into the exact treatment options. In an accredited laboratory, the standard demands that the laboratory actively provides advice on choice of treatment and clarification of any laboratory outcomes. As discussed previously, some decisions such as fertilizing oocytes using IVF or ICSI should be taken by a senior embryologist rather than a

doctor. The ultimate approach is to have the couple/patient sit down with the embryologist after oocyte and sperm collection for a “post-oocyte pick up (OPU) chat.” This gives the opportunity for the embryologist to discuss with the couple/patient issues such as the quality and numbers of sperm and oocytes and advise them on the best procedure ahead. This short chat should also include reminding the couple/patient of risk and success; that is, there is always a risk for failed fertilization, failed cleavage, or failed blastocyst development. If the couple/patient has been reminded of these risks, it makes it somewhat less stressful to make a call to them in the unlikely event of a failed fertilization.

Evaluations and audits

(ISO 15189:2022; 8.8)

Audits can be internal or external, vertical or horizontal, or process-oriented or system-oriented. Therefore, it is easy to get confused and caught up in terminology and to miss out on the great opportunity that audits provide for improving the system and our service to patients. To find nonconformities at an audit is not bad—it is proof that the system is working and we are capable of recognizing our weaknesses and faults and ready to learn and improve on them. For general internal audit principles, see Chapter 32 [7].

Internal audits

The laboratory standards are more precise in what exactly should come out of an audit and what is needed for a correct audit process. When preparing, writing, and implementing internal audit procedures, ISO 15189 is precise on what exactly is needed. The current standard requires laboratories to take a risk-based approach to audit intervals with processes which are high risk to patients be audited frequently. Poor outcomes of previous audits also require a specific process to be audited more frequently to assure that the corrective action has been efficient.

External audits

If the laboratory aims to seek formal accreditation to ISO 15189, the National Authority for Conformity Assessment performs the external audits. A formal accreditation is always advantageous, but in many countries, this option is not available, and as it is a rather pricey process, some laboratories choose to state that they adhere to the standard without formal accreditation.

When a laboratory is ready to be formally accredited, they need to apply for accreditation and the national authority will assess whether they have the appropriate expertise to perform the audit. If not, they can seek help from other members of the International Laboratory Accreditation Cooperation (ILAC) [74] or European Accreditation [75] who have the appropriate experienced auditors. Together with the application, the laboratory has to supply evidence of a fully compliant quality system and it is essential that all methods for which accreditation is sought have gone through a series of internal audits. Result documentation from these audits is supplemental to the application. The accreditation body then arranges a pre-audit to assess the readiness of the laboratory, and, pending the outcome of this pre-audit, an accreditation audit will be arranged. When the accreditation audit has been done, the lead auditor or any technical experts can only recommend that the laboratory be awarded accreditation. This recommendation is then passed on to the board of the accreditation body, which will decide if the laboratory is to be awarded accreditation.

Beyond the standards

While the embryology laboratory could be seen as any other clinical medical laboratory, there are some major differences to do with the delicacy of the samples it handles. Whereas a mistake in the day-to-day pathology laboratory can mostly be rectified by resampling, a mistake in the embryology laboratory can lead to major irreparable trauma for the patients [60]. Therefore, it is of great importance that we acknowledge these differences and implement processes that help safeguard us from incidents. Although some national and regional guidelines acknowledge these differences, IVF laboratories worldwide need to understand and address this. There are three major areas concerning not only the safeguarding of patients' gametes and embryos but also aiming to protect the embryologists working in the laboratory: (i) training of embryologists to make sure that the staff handling these delicate samples and undertaking the complex IVF processes are properly trained, (ii) appropriate sample identification processes, and (iii) implementation of risk management processes.

Training and accreditation of embryologists

Personnel (ISO 15189:2022; 6.2)

Clinical embryology is a highly skilled profession, and the main contributors to IVF success are the skills and knowledge of the embryologists. When considering the impact that the training of embryologists has on results, it is evident that there is a need for formalized training programs in every clinical IVF laboratory.

When looking at the international ISO standards, the requirement for personnel is not clearly defined. The standard states that the laboratories need to specify the competence requirements for all processes and activities including requirements for education, qualification, training, re-training, technical knowledge skills, and experience (6.2.2a). Further, areas of responsibility should be clearly outlined together with duties in the documented job descriptions. There should be clearly documented procedures in place for the introduction and training of new staff and the re-introduction of staff after long periods of absence or leave, together with documentation on how proof of competence is issued. The management of the laboratory should formulate goals for each member of staff with respect to continuing education and professional development (6.2.4). These goals should be assessed and discussed at annual appraisals, which should be documented but kept confidential.

In recent years, there has been an increased focus on the training and accreditation/certification of clinical embryologists. A formal training programme has been in place for embryologists in the UK since 1995. The original program was provided through Association of Clinical Embryologists (ACE, the ACE Certificate) including a minimum of two years training with both practical and theoretical components. In 2019, ACE voted to merge with the Association of Biomedical Andrologists and the British Andrology Society with the Association of Reproductive and Clinical Scientists (ARCS) formed in 2020. ARCS is a unified professional society covering all aspects of reproductive science and research and is now the driving force behind embryology professional development [76]. Trainee embryologists enrol through a training program managed under the National Health Service (NHS) Scientist Training Program (STP) [77]. This is a three-year graduate entry program that is covered by a fixed-term employment and, upon finalization, awards the holder a master's degree in reproductive science from an accredited university. Post STP

training, clinical embryologists follow a career pathway towards registration through either the Academy for Healthcare Science (AHCS) or the Association of Clinical Scientists (ACS) [76]. After registration, embryologists can pursue membership of the Royal College of Pathologists.

In 2008, ESHRE introduced a certification for embryologists with the aim of certifying the competence of clinical embryologists working in IVF and of developing a formal recognition for embryologists [78]. It provides two different pathways to certification: a clinical embryologist track open for embryologists with at least a BSc degree in natural/life sciences, at least three years hands-on experience with human gametes and embryos in an ART laboratory, and a minimum of 50 hands-on core embryology procedures and a senior clinical embryologist track for candidates with either an MSc or PhD, at least six years hands-on experience in an ART laboratory, and a minimum of 50 hands-on core embryology procedures. All ESHRE members who meet the requirements can apply. The assessment includes a logbook outlining the procedures included in the training and the minimum cases done and passing a multiple-choice examination. The certification process is validated and recognized in accordance with Union Europe'enne des Me'decins Sp'e'cialistes (UEMS) and their Council for European Specialists Medical Assessment (CESMA). ESHRE also offer a continuous embryology education credit system, with the credits being needed for three-yearly renewal of the certificate. In the 10 years from its start in 2008, the program has certified 773 clinical and 493 senior clinical embryologists. In 2012, the certification was opened up for non-European candidates, and in 2018, a pilot long-distance on-site exam for a small group of 22 candidates was organized in India, simultaneously with the main exam that was held in Geneva [79]. The 2020 exam was cancelled due to the COVID 19 pandemic, and in 2021, the exams were held online for the first time.

In Canada, CFAS has issued guidelines for an applied training program and evaluation and development of competencies for ART laboratory professionals. While CFAS is not a certifying or regulatory body, the CFAS program aims to develop standards that all ART laboratory professionals should conform to and to verify individuals are up to those standards [56].

In the United States, the Practice Committees of the ASRM) and the SRBT have recently published comprehensive guidelines for human embryology, andrology, and endocrinology laboratories [58]. These guidelines clarify embryology laboratory staff minimum requirements for education, training, continuing education, and experience together with recommended minimum staff numbers to ensure safe operations.

In Australia, Scientists in Reproductive Technologies (SIRT) are in the process of formalizing embryology training, aiming for a future certification and continuous professional development system.

With the ever widening availability of the ESHRE certification and their exams on clinical embryology being the most widely accepted tests of knowledge from laboratory science in ART, there is still a need for clinics to find ways of formalizing training for their embryologists. Every clinic should have documented training procedures clearly stating the minimum of supervised procedures a trainee has to undertake before being signed off for independent work. For the ESHRE certification, this includes 50 procedures of each of OPU, semen analysis and preparation, insemination, ICSI, zygote and embryo evaluation, ET, cryopreservation of oocytes/embryos, and thawing of oocytes/embryos. Obviously, the outcome of those procedures needs to

be evaluated too, and the trainees have to meet the set KPIs of the clinic to be approved. To ensure the theoretical component—that the trainee knows why and not only how—it is suggested that essays set on subjects such as preimplantation genetic diagnosis and embryo development are included along with a small examination. It is also crucial to fulfil the need for continued professional development, allowing embryologists to attend conferences and workshops and to participate in research.

Sample identification, witnessing, and prevention of misidentification

One of the most crucial tasks in the IVF lab is to ensure the correct identity of gametes and embryos. Over the years, there have been numerous reports of misidentification resulting at best in a cancelled cycle if the mistake is identified before embryo transfer and at worst in tragedy if realized after the embryo transfer or indeed birth. These errors are generally the result of trained personnel not following the known procedure for reasons such as distraction, tiredness, or being rushed [80, 81]. Alternatively, it is caused by poorly written or non-existent policies and protocols (active failure vs. latent condition). The solution to misidentification is the development of robust identification procedures that are risk assessed (for further details, see the “Risk identification, management, and prevention” section).

The EU tissue directive includes demands for appropriate sample identification with the core being a unique identifier for each sample. However, the most stringent guidelines involving safe sample identification procedures are provided by the HFEA CoP [34]. In the UK, it is a licensing requirement to have robust ID systems (Mandatory Requirement T71, HFEA CoP), and all IVF laboratories must put in place processes to ensure that no mismatches of gametes or embryos or identification errors occur. With this comes a demand for double witnessing of the identification at all critical prints of the IVF laboratory process. The witnessing has to be signed at the time of the checked step, and records must be kept in each patient's case file. Together with this license requirement, the guidelines stipulate that all samples of gametes and embryos be labelled with at least the patient's full name and two of the following identifier: date of birth, hospital number, NHS number/Community Health Index (CHI) number or unique donor identifier. It is important to note that a patient's name or date of birth is not a unique identifier. The witnessing is mandatory and required every time gametes or embryos change vessel (dish or tube), and the person checking should have a full understanding of the process they are witnessing, allowing only trained clinic staff named on the HFEA license to undertake the check. At semen sample handover, oocyte retrieval, and embryo transfer, the patient is required as an active participant in the identification.

In Australia, the RTAC CoP Critical Criterion 7 on Identification and traceability sets out the requirements with a minimum of three forms of identification used to ensure the traceability of all persons and specimens [5]. Double witnessing is strongly recommended but not mandatory; rather, the CoP requires the laboratories to annually audit and risk assess the process. The technical bulletin on Patient and Sample Identification (Technical Bulletin 4) is very detailed and provides robust guidelines for identification; however, it is not enforceable [37]. Similarly, the ESHRE laboratory guidelines include a section on identification of patients and traceability of their reproductive cells [32].

While most laboratories use manual double witnessing, identification checks can also be electronic, with several witnessing

systems being available for embryology purposes. The technology applied includes radiofrequency technology and barcodes. The advantages of automated systems are that their accuracy is not affected by lack of concentration or poor protocols [81], and they have a significantly lower error rate than human error (0.001% compared with 1%–3%) [82]. So by introducing electronic witnessing, we can possibly reduce errors in misidentification and potentially add an extra level of patient safety [82, 83]. A recent study showed that apart from improving safety, applying an electronic witnessing approach could also improve timing and efficacy of processes [84]. From a patient perspective, improved ID processes and risk minimization through electronic witnessing is welcomed. In a survey of 408 patients undergoing IVF, more than 90% were concerned about errors and 92% of them confirmed that the introduction of electronic witnessing would reduce their concern about a biological mix-up in the laboratory [85].

However, it is important to underline that all the current electronic witnessing systems are based on some type of sticker being attached to the tubes and dishes and mistakes can certainly occur in printing and labelling. Moreover, while the systems are not foolproof, they are expensive and some are bulky, taking up a substantial space. The development of electronic witnessing systems for IVF is only at its infancy, and the technology will more than likely be refined in the future.

In addition to the HFEA CoP, RTAC bulletin, and ESHRE guidelines, which are IVF specific, there are several standards and recommendations on the subject of patient and sample identification. The CLSI guideline on Accuracy in Patient and Sample Identification [83] describes the essential components of processes and systems that need to be implemented for accurate patient and sample identification. It covers the whole process from the pre-examination phase to the reporting of results, underlining the importance of staff training, risk assessment, and the use of unique identifiers, and it relates to both manual and electronic systems. The previously mentioned “nine-patient safety solutions” from WHO/JCI have patient identification at its core [23]. The ISO standards also have demands of correct sample labelling; however, they offer little information on safe solutions.

There are certainly huge advantages to the use of manual double witnessing, but there is always a slight risk that a procedure like this can cause mistakes, as we cannot double the embryologist workforce. One major source of incidents in the IVF laboratory is insufficient staffing, and to be interrupted while working with embryos can have disastrous consequences. In a busy IVF lab setting, scientists need to switch repeatedly between the patients' material they are working on and the patients' material they are being asked to check [86, 87]. In practice, the principal operator interrupts their workflow to locate a “witness” and the “witness” is interrupted from their own task to carry out the double check. Daniel Brison [88] estimated that, in a well-staffed IVF lab, each embryologist was witnessing 15–20 other procedures in a morning on top of their own workload. Many laboratories today have very few embryologists, and with a witnessing routine in place, this will not only increase the workload but also add a heightened risk of distraction when an embryologist has to interrupt others' work to get them to witness a certain step in the procedure. Moreover, human beings and systems under stress will underperform in rushed situations and stress is known to affect human performance in many sectors, including the IVF laboratory. Most clinics have periods when patient throughput is increased without compensation in relation to staffing levels. Systematic overtime, overloaded work schedules, high cognitive

loads, and chronic staff shortages contribute to error-inducing environments [89–91]. In addition, other forms of stress such as inadequate training and lack of guidance have been identified as sources of identification errors [92].

When introducing a robust, safe ID system in the laboratory, the best way of starting is to avoid reinventing the wheel. Even if your laboratory is located outside the UK, the HFEA CoP Guidance note 18 provides a great guide on how to ensure that the correct gametes are mixed and the right embryos are transferred [34]. To make it simple, the IVF laboratory must have written protocols for witnessing and each step involved has to be risk assessed (documented). As a part of the standardization introduced into a laboratory, there will be written SOPs and flow charts, and it is easy to identify each step where a gamete or embryo changes tube or dish. Simply add a witnessing signature to the laboratory sheet to each of those steps (the procedure itself should already have a signature on the sheet). An exception to the witnessing requirements is the so-called forced functions, such as when a clinic receives only one sperm sample on a given day, and so, there will be a forced function when the sample is transferred from one tube to another. If the clinic makes use of this, it has to be risk assessed.

With the first step in the process being reception of gametes, semen samples, or oocytes and the last step being embryo transfer, the HFEA CoP underlines the need for the patient to be involved in this crucial identification step. Here, it is important to implement a process that involves positive patient identification, which is the foundation for error prevention [93]. In simple words, the embryologist will ask the patient to audibly read out his/her name and any other identifier you have chosen such as date of birth and at the same time have a witness—the doctor or nurse—to confirm this positive identification step being done.

The witnessing action itself also needs to be done correctly. It should include three major components: (i) the ID-labelled vessel that holds the gametes or embryos; (ii) the new vessel that the sample is being moved in to, labelled with the same ID; and (iii) the patient documentation (i.e., the laboratory sheet containing the full identification of the patient). In addition to these three components, the embryologist performing the “move” (principle operator) reads the name and unique identifier aloud from the sample vessel, the new vessel, and the laboratory sheet, followed by the witness reading aloud the same.

Other hugely important factors are the strength and quality of the identifier itself. The need for a strong unique identifier together with the name is paramount. With the date of birth being too weak and not considered unique, the clinic or laboratory needs to create a couple-specific identifying number such as a unit number or couple number. A patient-specific number such as a medical records number is not advisable as the embryo mostly belongs to the couple and not one patient only. This identification, name, and couple number then need to be affixed to the vessel in a clear, safe manner. The most widely used labelling is handwriting with a nontoxic pen. Usually, the ID is written on the side of tubes and bottom of dishes (mirrored from the outside) to allow easy noticing. Printed stickers are also being used; however, it should be made clear that stickers contain glue and, when placed in a humidified incubator, this results in an increase of VOCs, which in turn can be toxic to the embryo. Another way of labelling is etching the ID into the plastic using a small syringe, but scratching of plastic will also increase VOCs and can be toxic to the embryo. Moreover, the etched details appear very faint and cannot be considered safe from a clear witnessing point of view.



FIGURE 5.1 Sperm preparation areas RED and BLUE each containing all equipment needed for complete sperm preparation. (a) Documentation for the patient, assigning work area RED to this patient. (b) Labelled sample pot and preparation tubes are double witnessed when brought into the area. (c) Only the sample currently being prepared in area RED is centrifuged in the area's designated centrifuge. When the preparation is complete, the area is sterilized before being assigned to the next patient.

Finally, the ID should always be affixed to the part of the vessel actually carrying the sample. Labelling the lid of a dish or a tube is not acceptable.

If a process involves gametes or embryos changing vessel several times during a short time period, such as sperm preparation or embryo freezing/thawing, then it can be acceptable to witness the whole area. For example, a laboratory preparing sperm can have multiple biosafety cabinets with one designated centrifuge and other equipment assigned to a specific defined work area. Note that each work area must have a designated centrifuge and two samples cannot be centrifuged together if this approach is adapted. When a sample is being brought into this area, all tubes involved can be witnessed at the same time with the prospect that only one sample will be handled through the whole process from start to finish (Figure 5.1). Obviously, this process needs to be risk assessed if adapted.

Correct labelling together with witnessing procedures will help minimize the risk of misidentification, but it is also absolutely imperative that only one couple's samples are handled at any one time. Preparation of a number of sperm samples, or cryopreservation or thawing of multiple patients' embryos at the same time, poses a huge risk for mix-ups and should never be done.

Risk identification, management, and prevention

According to the WHO, one in six couples experience difficulties in conceiving and would need some form of assisted reproduction method [94]. Worldwide, more than eight million children had been born as a result of an ART treatment in 2019, and it is estimated that over two-and-a-half-million treatment cycles are undertaken annually, resulting in 500,000 deliveries every year, possibly taking that number to more than 10 million at the time of writing [95]. With the increase in IVF cycle number worldwide, it has become evident that just like in other areas of medicine and healthcare, errors are inherent. But it is important to remember that these errors most often result from a complex interplay of multiple factors; only rarely are they due to the carelessness or misconduct of single individuals. Historically, rather than addressing the source of errors, prevention strategies have relied almost exclusively on enhancing the carefulness of the caregiver [96]. A culture of blame and finding

a scapegoat has commonly been the response to adverse events, and this is an approach that can never improve the system and prevent the incident from reoccurring. The portioning of blame to an individual usually comes with a promise that "it will never happen again" [97]. The crucial changes in the approach to risk management in IVF clinics are presented in Table 5.1. In order to prevent errors and identify risks, IVF laboratories must introduce robust risk management including an analysis of systems and structures in advance of those risks actually materializing, thus embedding risk management into the daily routine for embryologists. The international standard ISO 31000:2018 Risk Management Guidelines [98] is the most widely acknowledged tool for addressing, managing, and preventing risk. Implementing this standard will not only vastly decrease the risk of adverse events and near misses but also provide tools for how to learn from incidents when they happen and prevent them from happening again. ISO 31000 will provide a clear guide on how to set up a risk management policy and clearly outlines what needs to be included.

Errors and incidents result from failures, and these can be categorized as active failures or latent conditions [99, 100]. Active failures result from violation of the agreed protocols, lapses, or mistakes. Latent conditions or errors include error-provoking conditions such as workload, fatigue, knowledge, supervision, and equipment and weaknesses in defence including unworkable procedures or switching off a malfunctioning alarm. Latent conditions are embedded in all systems as it is not possible to foresee all error-producing situations. However, as they pre-exist, active failures may be able to be identified prior to adverse events occurring. Therefore, these conditions tend to be the targets of risk management systems.

The first step towards risk management in the laboratory is to have a clear overview of the protocols and procedures undertaken by the embryologists. This should be provided already as part of the quality system and demand for SOPs. With the use of process maps and flow charts for the procedures, it will be easy to identify areas and procedures that could be high risk, but total risk management has to include all processes and procedures. Mortimer and Mortimer [101] provide a simple summary of risk management by asking and answering three basic questions: what can

TABLE 5.1 Shift in Approaches to Risk Management in *In Vitro* Fertilization Clinics

Outdated Approach	Modern Approach
Main goal	
To protect the IVF clinic's reputation	To improve patient safety and minimize risk of harm to and misidentification of embryos and gametes through better understanding of systemic factors that affect the risk for incidents
Reporting	
Acknowledge only reports submitted in writing	Variety of methods to report: paper form, electronic form, telephone call, anonymous reporting, and person-to-person reporting
Investigation	
Investigate only the serious occurrences	Encourage reporting of "near misses" and investigate and discuss the potential causes
Interview staff one on one when there is an adverse incident	Have root cause analysis meetings with the entire team
Corrective/preventive action	
Blame and train (or dismissal)	Perform a criticality analysis chart and determine the root cause of the "near miss" or the adverse occurrence
Work with department involved to develop corrective action	Work with the team to develop a safety improvement plan
Information from investigation kept confidential	Develop corrective action and share with the whole IVF team
Communication	
Talk to the patients only if necessary and be vague about incident/findings	Advise clinic director to speak directly with the patients and talk with them about any unexpected outcome and error; keep them apprised of steps taken to make the environment safe for the next patient
Long-term follow-up	
Assume that action is taken to correct the problem that occurred, and notice only when it happens again that no action is taken	Monitor and audit to determine that changes have been initiated and that the changes have made a difference

Source: Adapted from [96].

Abbreviations: IVF, *in vitro* fertilization.

go wrong? What will we do? If something happens, how will we resolve it? There are three core tools for helping us address risk and to answer those questions: failure mode and effects analysis (FMEA), root cause analysis, and audit.

A comprehensive way of proactively addressing risk is to make use of FMEA. Like many approaches that improve quality and safety, FMEA has its origins in the army, space, and aviation industry, but it is now used as a tool for error prevention in a wide range of industries, including healthcare. The aim of FMEA is to try to think of every possible way a process can go wrong, how serious it would be, and how the process can be improved to avoid failure. It is important that all embryologists on the team are involved in assessing each process using FMEA. A simple format for FMEA is illustrated in [Table 5.2](#). The first step is to identify the process to be assessed, using the examples of insemination, mixing of oocytes, and sperm for IVF. Then, identify what could go wrong (potential failure mode); for example, an embryologist forgets to inseminate, mixes the wrong oocytes and sperm, loses oocytes, and bumps a dish. Then, ask “what could be the

result of this failure?" It could be failed fertilization, creation of an embryo or indeed child with the "wrong parents," and decreasing the chances of pregnancy. Then, assess the seriousness of the suggested failures using a 1–10 scale with 1 being no effect and 10 being critical. For failed fertilization, one could argue a seriousness of 8, but the creation of a mixed-up embryo has a severity of 10. Once severity has been established, address the different causes of the failure; in this case, being rushed, low staffing levels, poor processes, lack of checklists, and no witnessing system. Then, rate how often this would happen, from 1 being no known occurrences (has never happened in any IVF clinic) and 10 being very high risk (with this happening regularly). Forgetting to inseminate happens in all clinics, but one could argue that it is very rare, so an occurrence of 2 or 3 would be appropriate. Then, discuss and list the current controls (e.g., use of daily worksheets or reminders) followed by assessing what chance there is that we would detect the failure. With forgetting to inseminate, this will be evident the morning after when the oocytes are found without sperm and are unfertilized, and we can assign this a 1 representing

TABLE 5.2 Sample Failure Mode and Effects Analysis Worksheet

detection every time it happens. However, with the case of a mix-up, this could go completely undetected and should be assigned a 9–10; the fault will be passed to the customer undetected, or, in IVF terms, the resulting embryo will be transferred leaving the patient or child to detect the failure. Then, calculate the risk priority number (RPN) by multiplying the severity, occurrence, and detection; for forgetting to inseminate, this is $8 \times 3 \times 1 = 24$. Now the initial analysis is done and the embryology team has the task of lowering the RPN. There needs to be an active discussion on how the procedure can be changed, allowing everyone on the team to come up with suggestions. Remember that we can sometimes grow accustomed to our own best practices but should consider the suggestions from trainees, who after all provide us with a fresh pair of eyes. Preventing failure in insemination could include the introduction of daily worksheets and checklists together with witnessing and improved ID checks. When these suggested changes have been discussed, documented, and implemented, a new value for severity, occurrence, and detection is assigned and the new RPN should hopefully be significantly lower than the original.

The FMEA exercise is not only a mathematical exercise resulting in reduced risk through the actions taken but also a great way of making all embryologists aware of what risks are involved in each step of the IVF process, and this awareness itself can help reduce risks. There are some excellent studies published on applying FMEA to embryology laboratory procedures including core embryology processes [102, 103], PGT [104], and witnessing [105].

Even the best risk management systems have incidents and near misses. So, what can be done when an incident occurs? The answer is root cause analysis, which is the reactive component in a risk management system. A root cause analysis is simply an analysis of the very reason for the incident occurring. A simple example is when recently trialling a new incubator, the lid accidentally fell over the hand of the embryologist while placing dishes inside, resulting in spillage of the medium and loss of 1 out of 23 oocytes. The root cause analysis included discussing the incident at the lab meeting: Had it happened before? Were there any near misses previously where the lid had been falling without incident? But also we discussed how we place dishes in the incubators: Are we sometimes carrying more than one dish? We further contacted the supplier to see whether it was a fault of the incubator itself. It was concluded that the lid of our trial incubator did not recline and was a risk if left open without holding on to it. We implemented a procedure where only one dish could be carried and placed in the incubator at any one time, always allowing one hand to be free to hold up the lid. At no time is it appropriate to revert to the old, outdated way of thinking where we apportion blame; this can never result in improvement. More complex root cause analyses could focus on the failure to inseminate as used for the FMEA, instead of looking at it proactively, doing a root cause analysis after the fact. Mortimer and Mortimer [101] provide an interesting example of root cause analysis of poor fertilization results, with the outcome being a complete reformulation of the fertilization medium.

Many root cause analyses I have been involved in concluded that the level of staffing was inappropriate. It is important to underline that staffing issues such as overworking and poor training are the main contributors to incidents. There is also the issue with staff who are not accepting professional responsibility and do not take enough care to undertake their duties or follow protocols; they should not continue to work in the laboratory [101].

Finally, a very effective tool in addressing and analysing risk is audits. All incidents followed by a root cause analysis will include suggestions for change and continuous improvement. To ensure these have been implemented and are indeed effective, one needs to undertake internal audits (see the “Evaluations and audits” section).

Another side to safe practice is to have robust contingency plans. There should always be a documented, agreed plan B. This will include having a backup for all equipment, such as a minimum of two microscopes, heated stages, and centrifuges. For more expensive equipment such as ICSI rigs, oocyte aspiration pumps, and controlled freezers, where sometimes the clinic cannot afford to have two sets, there needs to be a written agreement with another IVF clinic regarding utilization of their equipment.

Concluding remarks and future aspects

Throughout completing the long and work-intensive process of applying standardized systems in an embryology laboratory, one might ask what it has meant for the embryologists and the results of the clinic. There is no doubt that introducing and fully implementing a quality system standardizes methods and the ways in which embryologists perform their work. The troubleshooting, maintenance of equipment, and milieu are improved and standardized. This guarantees optimal handling of a couple’s gametes and embryos and inevitably will lead to improved outcome.

The number of ART treatment cycles undertaken worldwide is increasing every year, and with the improvement of the techniques we use, more babies are born as a result of IVF. With the outcome improving, we are aiming towards a future where more focus will be on the safety of treatment and indeed the long-term health of children resulting from ART. With this comes a demand for standardization and improvement of quality. The introduction of quality management systems will ensure reproducibility and traceability, which will be crucial for the future follow-up of these children.

To face the future, we need to improve our understanding of the long-term effects of our laboratory procedures on embryo health, acknowledging that some of our methods might deliver in numbers but might be detrimental when considering the adult health of children conceived through IVF. A review of the follow-up of children born from IVF over 25 years in Sweden has revealed that in contrast to cleavage-stage transfer, children born after blastocyst transfer exhibited a higher risk of preterm birth and congenital malformations [106]. A study comparing euploidy rates in donor egg cycles between different fertility centres showed some centres achieving euploidy rates between 70% and 80% while other centres having rates as low as 40%–50% [107]. Considering the nature of donated oocytes as somewhat standardized, these results strongly indicate that there are laboratory practices that contribute to higher or lower euploidy rates. Taking these two studies into account, it is evident that suboptimal culturing and handling of embryos have long-reaching effects far beyond blastocyst development, successful pregnancy, and live birth. It indicates that what we do in the clinical embryology laboratory is closely connected to the adult health of children born from IVF. This further highlights the importance of standardization, along with implementing processes that go beyond the standards, working towards improved risk management, robust and thorough training of clinical embryologists, and processes to ensure correct identification and prevention of mix-ups.

Finally, it is important to acknowledge that quality management together with a never-ending commitment to improve our service, beyond standards, is the only way forward towards a future where we can guarantee safe, efficient IVF treatment for all patients and the birth of children who go on to live healthy lives.

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References

1. Präg P, Mills MC. Assisted reproductive technology in Europe: Usage and regulation in the context of cross-border reproductive care. In *Childlessness in Europe: Contexts, Causes, and Consequences*. Kreyenfeld M, Konietzka D (eds.). Cham: Springer, pp. 289–309, 2017.
2. Dawson KJ. Quality control and quality assurance in IVF laboratories in the UK. *Hum Reprod.* 1997;12(12):2590–1.
3. Pool TB. Practices contributing to quality performance in the embryo laboratory and the status of laboratory regulation in the US. *Hum Reprod.* 1997;12(12):2591–3.
4. Lieberman BA, Matson PL, Hamer F. The UK Human Fertilisation and Embryology Act 1990—how well is it functioning? *Hum Reprod.* 1994;9(9):1779–82.
5. Code of Practice for Assisted Reproductive Technology Units. FertilitySocietyofAustraliaReproductiveTechnologyAccreditation Committee (RTAC) 2021 [1/3/2022]. Available from: <https://www.fertilitysociety.com.au/wp-content/uploads/20211124-RTAC-ANZ-COP.pdf>.
6. ISO 9001:2015. Quality Management Systems—Requirements. 6th ed. Geneva: International Organization for Standardization; 2015.
7. Alper M. Quality management in reproductive medicine. In: *Textbook of Assisted Reproductive Techniques*. 6th ed. Gardner DK, Weissman A, Howles CM, Shoham Z (eds.). London: CRC Press, 2022.
8. ISO 17025:2012. General Requirements for the Competence of Testing and Calibration Laboratories. Geneva: International Organization for Standardization. 2005, 2012.
9. EN 45001. General Criteria for the Operation of Testing Laboratories. Geneva: International Organization for Standardization, 1989.
10. ISO/IEC Guide 25. General Requirements for the Competence of Calibration and Testing Laboratories. 3rd ed. Geneva: International Organization for Standardization., 1990.
11. Wiklund M, Sjöblom C. The application of quality systems in ART programs. *Mol Cell Endocrinol.* 2000;166(1):3–7.
12. ISO/DIS 15189(en) Medical laboratories — Requirements for quality and competence Geneva: International Organization for Standardization; 2022 [1/5/2022]. 4th ed. Available from: <https://www.iso.org/obp/ui/#iso:std:iso:15189:dis:ed-4:v1:en>.
13. Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells: Official J Eur Union. 2004;102:48–58 [1/3/2022]. Available from: <http://eurlex.europa.eu/Lex-UriServ/LexUriServ.do?uri=OJ:L:2004:102:0048:0058:en:PDF>.
14. Guide to the Quality and Safety of Tissues and Cells for Human Application: Council of Europe European Directorate for the Quality of Medicines & HealthCare (EDQM); 2019 [1/3/2022]. 4th ed. Available from: <https://www.edqm.eu/documents/52006/75920/transplantation+leaflet+guide+quality+safety+tissues+and+cells+human+application+4th+edition+2019.pdf?7804294d-81dd-2933-cla5-04fb152a4c87?t=1648045555391>.
15. Commission Directive 2006/17/EC of 8 February 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards certain technical requirements for the donation, procurement and testing of human tissues and cells (Text with EEA relevance): Official J Eur Union. 2006;38:40–52 [1/3/2022]. Available from: <http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32006L0017>.
16. Commission Directive 2006/86/EC of 24 October 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards traceability requirements, notification of serious adverse reactions and events and certain technical requirements for the coding, processing, preservation, storage and distribution of human tissues and cells (Text with EEA relevance): Official J Eur Union. 2006; 294:32–50 [1/3/2022]. Available from: <https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:294:0032:0050:EN:PDF>.
17. Commission Directive 2012/39/EU of 26 November 2012 amending Directive 2006/17/EC as regards certain technical requirements for the testing of human tissues and cells (Text with EEA relevance): Official J Eur Union; 2012; 327: 24–25 [1/3/2022]. Available from: <https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1463631863044&uri=CELEX:32012L0039>.
18. ESHRE position paper on the EU Tissues and Cells Directive EC/2004/23: ESHRE; 2007 [1/3/2022]. Available from: <https://www.eshre.eu/~media/sitecore-files/Guidelines/Guidelines/Position-Papers/Tissues-and-cells-directive.pdf?la=en>.
19. Proposal for a regulation of the European parliament and of the council on standards of quality and safety for substances of human origin intended for human application and repealing Directives 2002/98/EC and 2004/23/EC: Official J Eur Union; 2022/0216 (COD) [20/07/2023]. Available from: <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52022PC0338>.
20. Updating the EU Tissue & Cells Directives Focus on Reproduction: ESHRE;2020[1/3/2022].Availablefrom:<https://www.focusonreproduction.eu/article/ESHRE-News-Annual-Meeting-2020-Fehily>.
21. The EU's tissue and cell directives will shortly be superseded by a new single legislation; ESHRE outlines the 'key considerations': Focus on Reproduction; 2022 [20/07/2023]. Available from: <https://www.focusonreproduction.eu/article/News-in-Reproduction-SoHo>.
22. Joint Commission International Standards for Laboratories. fourth ed: Joint Commission International; 2021. Available from: www.jointcommissioninternational.org.
23. WHO Collaborating Centre for Patient Safety Solutions. Patient Safety Solutions [1/3/2022]. Available from: <http://www.ccforpatientsafety.org/Patient-Safety-Solutions>.
24. Clinical and Laboratory Standards Institute [1/3/2022]. Available from: <http://www.clsi.org>.
25. Reproductive Accreditation Program: College of American Pathologists (CAP); [1/5/2022]. Available from: <https://www.cap.org/laboratory-improvement/accreditation/reproductive-accreditation-program>.
26. Commission Directive 2003/94/EC, Laying down the principles and guidelines of good manufacturing practice in respect of medicinal products for human use and investigational medicinal products for human use: Official J Eur Union. 2003;14:L262/22-6 [1/3/2022]. Available from: <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32003L0094>.
27. EU GMP Annex 1: Manufacture of Sterile Medicinal Products - revision November 2008: European Commission; 2008 [1/3/2022]. Available from: [https://www.gmp-compliance.org/files/guidemgr/annex%2001\[2008\].pdf](https://www.gmp-compliance.org/files/guidemgr/annex%2001[2008].pdf).
28. WHO laboratory manual for the examination and processing of human semen. 6th ed. Bjorndahl L (ed.). Geneva: World Health Organization, 2021.

29. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Hum Reprod.* 2011;26(6):1270–83.
30. The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators. *Reprod Biomed Online.* 2017;35(5):494–510.
31. The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: proceedings of an expert meeting. *Reprod Biomed Online.* 2012;25(2):146–67.
32. Eshre Guideline Group on Good Practice in IVF Labs, De los Santos MJ, Apter S, Coticchio G, Debrock S, Lundin K, et al. Revised guidelines for good practice in IVF laboratories (2015)†. *Hum Reprod.* 2016;31(4):685–6.
33. Human Fertilisation and Embryology Authority (HFEA). Available from: www.HFEA.gov.uk.
34. Human Fertilisation and Embryology Authority (HFEA). Code of Practice 2019 [updated October 2021; 1/3/2022]. 9th edition. Available from: <https://portal.hfea.gov.uk/media/1756/2021-10-26-code-of-practice-2021.pdf>.
35. The Human Fertilisation and Embryology (Amendment) (EU Exit) Regulations 2020 No. 1307 legislation.gov.uk. United Kingdom Parliament; 2020 [23/05/2022]. Available from: <https://www.legislation.gov.uk/uksi/2020/1307/contents/made>.
36. Chair's letter 09/06/2021 - CH(21)02: Human Fertilisation and Embryology Authority (HFEA); 2021 [23/5/2022]. Available from: <https://portal.hfea.gov.uk/knowledge-base/chair-s-letters/3445>.
37. Reproductive Technology Accreditation Committee RTAC Technical Bulletins. Available from: <https://www.fertilitysociety.com.au/code-of-practice/>.
38. Chinese ART laws and regulations can be obtained by contacting the National Health and Family Planning Commission of the PRC. Available from: <http://en.nhc.gov.cn>.
39. Interpreting the regulations 2021: 关于 2021 版指导原则的解读: Chinese Society of Reproductive Medicine (CSRMM); 2021 [22/5/2022]. Available from: <http://www.nhc.gov.cn/fys/s3581/202101/68396c1041bc4326a1fdb2c0687dbf2f.shtml>.
40. Assisted Reproductive Technology (Regulation) ACT: Indian Ministry of Law and Justice; 2021 [1/5/2022]. Available from: <https://www.indiacode.nic.in/bitstream/123456789/17031/1/A2021-42%20.pdf>.
41. National Guidelines for Accreditation, Supervision & Regulation of ART Clinics in India. Indian Council of Medical Research National Academy of Medical Sciences (India). 2005 [1/3/2022]. Available from: https://main.icmr.nic.in/sites/default/files/guidelines/Prilim_Pages.pdf.
42. Malhotra J, Malhotra K, Talwar P, Kannan P, Singh P, Kumar Y, et al. ISAR Consensus Guidelines on Safety and Ethical Practices in In vitro Fertilization Clinics. *J Hum Reprod Sci.* 2021;14 (Suppl 1): S48–68.
43. Japanese Institution for Standardizing Assisted Reproductive Technology (JIS-ART). [1/5/2022]. Available from: <https://jisart.jp>.
44. Revised Licensing Terms and Conditions on Assisted Reproduction Services Imposed Under Section 6(5) of the Private Hospitals and Medical Clinics Act [Cap 248]: Ministry of Health Singapore. 2019. Available from: [https://www.moh.gov.sg/licensing-and-regulation/regulations-guidelines-and-circulars/details/revised-licensing-terms-and-conditions-on-assisted-reproduction-services-imposed-under-section-6\(5\)-of-the-private-hospitals-and-medical-clinics-act-cap-248](https://www.moh.gov.sg/licensing-and-regulation/regulations-guidelines-and-circulars/details/revised-licensing-terms-and-conditions-on-assisted-reproduction-services-imposed-under-section-6(5)-of-the-private-hospitals-and-medical-clinics-act-cap-248).
45. Code of Practice for Assisted Reproductive Technology Units, International Edition, Fertility Society of Australia Reproductive Technology Accreditation Committee. 2018 [1/3/2022]. Available from: <https://www.fertilitysociety.com.au/wp-content/uploads/2018-RTAC-International-COP-FINAL-1.pdf>.
46. Inhorn MC. Making Muslim babies: IVF and gamete donation in Sunni versus Shi'a Islam. *Cult Med Psychiatry.* 2006;30(4):427–50.
47. Ali A. The Conditional Permissibility of In Vitro Fertilisation Under Islamic Jurisprudence al-Ghazzali Centre Awareness Paper. al-Ghazzali Centre For Islamic Sciences & Human Development. Available from: www.alghazzali.org.
48. Federal Law No. (07) of 2019 Concerning the Medically Assisted Reproduction. Department of Health UAE, 2019 [22/5/2022]. Available from: https://mohap.gov.ae/app_content/legislations/php-law-en-96/mobile/index.html#p=1.
49. DOH Standard for Assisted Reproductive Technology Services and Treatment. Department of Health UAE, 2022 [22/5/2022]. Available from: <https://www.doh.gov.ae/-/media/1FF4AC8529104411A2D274670A76709B.ashx>.
50. Kooli C. Review of assisted reproduction techniques, laws, and regulations in Muslim countries. *Middle East Fertil Soc J.* 2019;24(1):8.
51. Normas para la acreditacion de centros de reproduccion asistida y sus laboratorios de embriologia y andrologia. Version 15 2020 [1/5/2022]. Available from: <https://redlara.com/images/arg/Normas-AC-version-15%20ago2020.pdf>.
52. Calculadora Raproducción Asistida CASSANDRA, Human resources calculator in assisted reproduction: ASEBIR, [1/5/2022]. Available from: https://asebir.com/cassandra-calculadora-de-rrhh/?idioma_cassandra=en.
53. Assisted Human Reproduction Act. S.C. c.2 Department of Justice Canada, 2004 [1/5/2022]. Available from: <https://laws-lois.justice.gc.ca/PDF/A-13.4.pdf>.
54. Assisted human reproduction. Health Canada, 2021 [1/5/2022]. Available from: <https://www.canada.ca/en/health-canada/services/drugs-health-products/biologics-radiopharmaceuticals-genetic-therapies/legislation-guidelines/assisted-human-reproduction.html>.
55. Guidance Document Safety of Sperm and Ova Regulations. Health Canada, 2021 [23/5/2022]. Available from: <https://www.canada.ca/en/health-canada/programs/consultation-safety-sperm-ova-regulations/document.html>.
56. Canadian Fertility and Andrology Society (CFAS), ART Lab Special Interest Group [1/5/2022]. Available from: <https://cfas.ca/art-lab-sig.html>.
57. Practice Committee of the American Society for Reproductive Medicine, Practice Committee of the Society for Assisted Reproductive Technology, and Practice Committee of the Society of Reproductive Biologists and Technologists. Minimum standards for practices offering assisted reproductive technologies: a committee opinion. *Fertil and Steril.* 2020;113(3):536–41.
58. Practice Committees of the American Society for Reproductive Medicine (ASRM) and the Society for Reproductive Biologists and Technologists (SRBT). Comprehensive guidance for human embryology, andrology, and endocrinology laboratories: management and operations: a committee opinion. *Fertil Steril.* 2022; 117(6):1183–202.
59. ISO 15189:2012. Medical laboratories—Requirements for Quality and Competence. 3rd ed. Geneva: International Organization for Standardization, 2012.
60. van Kooij RJ, Peeters MF, te Velde ER. Twins of mixed races: consequences for Dutch IVF laboratories. *Hum Reprod.* 1997; 12(12):2585–7.
61. Hartshorne GM, Baker H. Fads and foibles in ART: where is the evidence? *Hum Fertil (Camb).* 2006;9(1):27–35.
62. Takenaka M, Horiuchi T, Yanagimachi R. Effects of light on development of mammalian zygotes. *Proc Natl Acad Sci U S A.* 2007;104(36):14289–93.
63. Oosafe MEA tested IVF laboratory disinfectants. Denmark, Sparmed, Stenlose [1/5/2022]. Available from: <https://www.sparmed.dk/en/products/disinfectants/>.
64. Nijs M, Franssen K, Cox A, Wissmann D, Ruis H, Ombelet W. Reprototoxicity of intrauterine insemination and in vitro fertilization-embryo transfer disposables and products: a 4-year survey. *Fertil Steril.* 2009;92(2):527–35.
65. Council Directive 93/42/EEC of 14 June 1993 concerning medical devices, OJ L 169, 12/7/1993. Amended by Directive 2007/47/EC of the European Parliament and of the Council of 5 September 2007 (OJ L 247, 21.9.2007). [1/5/2022]. Available from: <https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1993L0042:20071011:en:PDF>.

66. Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices. OJ L 331, 7/12/1998. Amended by Commission Directive 2011/100/EU (OJ L 341, 22/12/2011) [1/5/2022]. Available from: <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A01998L0079-20120111>.
67. Liperis G, Sjöblom C. Quality control in the IVF Laboratory: continuous improvement. In: Morbeck DE, Montag MHM (eds). Principles of IVF Laboratory Practice: Optimizing Performance and Outcomes. Cambridge: Cambridge University Press, 2017, pp. 79–87.
68. Janssens R, Munck ND, Guns J. Quality control maintaining stability in the laboratory. In: Gardner DK, Weissman A, Howles CM, Shoham Z (eds). Textbook of Assisted Reproductive Techniques. 6th ed. London: CRC Press, 2022.
69. ESHRE Clinic PI Working Group; Vlaisavljevic V, Apter S, Capalbo A, D'Angelo A, Gianaroli L, et al. The Maribor consensus: report of an expert meeting on the development of performance indicators for clinical practice in ART. *Hum Reprod Open*. 2021;2021(3):hoab022.
70. United Kingdom National External Quality Assessment Service (UK NEQAS), 2023. [1/5/2022]. Available from: <https://ukneqas.org.uk/>.
71. GameteExpert, 2010. [1/5/2022]. Available from: <https://gamete-expert.com/>.
72. QAP online FertAid, 2002. [1/5/2022]. Available from: <http://www.fertaaid.com/>.
73. ISO/IEC 17043:2010 Conformity assessment—General requirements for proficiency testing. Geneva: International Organization for Standardization, 2010.
74. International Laboratory Accreditation Cooperation (ILAC), 2023. [1/5/2022]. Available from: <https://ilac.org/>.
75. European Accreditation. [1/5/2022]. Available from: <https://euro-pean-accreditation.org/>.
76. The Association of Reproductive and Clinical Scientists (ARCS). [1/5/2022]. Available from: <https://www.arcscientists.org/>.
77. The National Health Service (NHS) Scientist Training Programme (STP). [1/5/2022]. Available from: <https://www.healthcareers.nhs.uk/explore-roles/life-sciences/reproductive-science/training-development-and-registration>.
78. European Society of Human Reproduction and Embryology (ESHRE) certification for clinical embryologists, 2021. [1/5/2022]. Available from: <https://www.eshre.eu/Accreditation-and-Certification/Certification-for-embryologists.aspx>.
79. Kovačić B, Prados FJ, Plas C, Woodward BJ, Verheyen G, Ramos L, et al. ESHRE Clinical Embryologist certification: the first 10 years. *Hum Reprod*. 2020;2020(3):hoaa026-hoaa.
80. Technology Solutions to Patient Misidentification - Report of Review: Australian Commission on Safety and Quality in Health Care, 2008 [1/5/2022]. Available from: <https://www.safetyandquality.gov.au/sites/default/files/migrated/19794-TechnologyReview.pdf>.
81. Lusky K. Patient ID systems offer smart start. CAP Today. 2005 [1/5/2022]. Available from: http://www.captodayonline.com/Archives/feature_stories/1005_Patient_ID_systems.html.
82. Aller R. Positive patient identification: more than a double check (Positive patient identification systems and tools). CAP Today. 2005 [1/5/2022]. Available from: http://www.captodayonline.com/Archives/surveys/1005_System_Survey.pdf.
83. CLSI. Accuracy in Patient and Specimen Identification. 2nd ed. CLSI standard GP33. Wayne, PA: Clinical and Laboratory Standards Institute, 2019.
84. Holmes R, Wirkka KA, Catherino AB, Hayward B, Swain JE. Comparison of electronic versus manual witnessing of procedures within the in vitro fertilization laboratory: impact on timing and efficiency. *Fertil Steril*. 2021;2(2):181–8.
85. Forte M, Faustini F, Maggiulli R, Scarica C, Romano S, Ottolini C, et al. Electronic witness system in IVF-patients perspective. *J Assist Reprod Genet*. 2016;33(9):1215–22.
86. Kerr A. A problem shared ...? Teamwork, autonomy and error in assisted conception. *Soc Sci Med*. 2009;69(12):1741–9.
87. Adams S, Carthey J. IVF witnessing and electronic systems. HFEA commissioned report comparing the relative risks of witnessing systems, 2006.
88. Brison D. Reducing risk in the IVF laboratory: implementation of a double witnessing system. *Clinical Risk*. 2004;10(5):176–80.
89. Amalberti R, Auroy Y, Berwick D, Barach P. Five system barriers to achieving ultrasafe health care. *Ann Intern Med*. 2005;142(9):756–64.
90. Leape LL, Berwick DM. Safe health care: are we up to it? *BMJ*. 2000;320(7237):725–6.
91. Toft B, Mascie-Taylor H. Involuntary automaticity: a work-system induced risk to safe health care. *Health Serv Manage Res*. 2005;18(4):211–6.
92. Kennedy CR, Mortimer D. Risk management in IVF. *Best Pract Res Clin Obstet Gynaecol*. 2007;21(4):691–712.
93. Lippi G, Blanckaert N, Bonini P, Green S, Kitchen S, Palicka V, et al. Causes, consequences, detection, and prevention of identification errors in laboratory diagnostics. *Clin Chem Lab Med*. 2009;47(2):143–53.
94. Edi-Osagie E, Hooper L, Seif MW. The impact of assisted hatching on live birth rates and outcomes of assisted conception: a systematic review. *Hum Reprod*. 2003;18(9):1828–35.
95. Fauser BC. Towards the global coverage of a unified registry of IVF outcomes. *Reprod Biomed Online*. 2019;38(2):133–7.
96. Kuhn AM, Youngberg BJ. The need for risk management to evolve to assure a culture of safety. *Qual Saf Health Care*. 2002;11(2):158–62.
97. Wu AW. Medical error: the second victim. The doctor who makes the mistake needs help too. *BMJ*. 2000;320(7237):726–7.
98. ISO 31000:2018 Risk management—Guidelines. 2nd ed. Geneva: International Organization for Standardization, 2018.
99. Reason J. The contribution of latent human failures to the breakdown of complex systems. *Philos Trans R Soc Lond B Biol Sci*. 1990;327(1241):475–84.
100. Reason J. Human error: models and management. *BMJ*. 2000;320(7237):768–70.
101. Mortimer ST, Mortimer D. Quality and risk management in the IVF laboratory. Cambridge University Press, 2015.
102. Intra G, Alteri A, Corti L, Rabellotti E, Papaleo E, Restelli L, et al. Application of failure mode and effect analysis in an assisted reproduction technology laboratory. *Reprod Biomed Online*. 2016;33(2):132–9.
103. Rienzi L, Bariani F, Dalla Zorza M, Albani E, Benini F, Chamayou S, et al. Comprehensive protocol of traceability during IVF: the result of a multicentre failure mode and effect analysis. *Hum Reprod*. 2017;32(8):1612–20.
104. Cimadomo D, Ubaldi FM, Capalbo A, Maggiulli R, Scarica C, Romano S, et al. Failure mode and effects analysis of witnessing protocols for ensuring traceability during PGD/PGS cycles. *Reprod Biomed Online*. 2016;33(3):360–9.
105. Rienzi L, Bariani F, Dalla Zorza M, Romano S, Scarica C, Maggiulli R, et al. Failure mode and effects analysis of witnessing protocols for ensuring traceability during IVF. *Reprod Biomed Online*. 2015;31(4):516–22.
106. Finnström O, Källén B, Lindam A, Nilsson E, Nygren K-G, Olausson PO. Maternal and child outcome after in vitro fertilization—a review of 25 years of population-based data from Sweden. *Acta Obstet Gynecol Scand*. 2011;90(5):494–500.
107. Munné S, Alikani M, Ribustello L, Colls P, Martínez-Ortiz PA, McCulloh DH, et al. Euploidy rates in donor egg cycles significantly differ between fertility centers. *Hum Reprod*. 2017;32(4):743–9.

6

EVALUATION OF SPERM

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Introduction

Abnormalities in sperm production or function, alone or in combination with other factors, account for 40% of all cases of infertility. Although a battery of tests and treatments have been described and continue to be used in the evaluation of female infertility, the male has been essentially neglected. The majority of programs offering advanced assisted reproduction technologies (ART) employ only a cursory evaluation of the male—rarely extending beyond semen analysis. Several factors account for this disparity. First, most practitioners of ART are gynaecologists or gynaecologic subspecialists who have little formal training in the evaluation of infertile or sub-fertile men. Second, the urologists, who perhaps theoretically should have taken the lead in this area, have devoted little of their literature or research budgets to the evaluation of the infertile male. Third, and perhaps most important, is the inescapable fact that sperm function testing remains a very controversial area of research. Many tests have been described, yet few have been extensively evaluated in a proper scientific manner. Those that have continue to be criticized for poor sensitivity or specificity, a lack of standardization of methodology, suboptimal study design, problems with outcome assessment, and the lack of long-term follow up. Although many of these same criticisms could also be levelled against most diagnostic algorithms for female infertility, in that arena, the tests continue to prevail over their critics. Fourth, like female infertility, male infertility is certainly multifactorial. It is improbable that one sperm function test will prove to be a panacea, owing to the multiple steps involved in fertilization. In addition to arriving at the site of fertilization, sperm must undergo capacitation and the acrosome must allow for the penetration of the cumulus cells and the zona pellucida so the sperm head can fuse with the oolemma. In addition, the sperm must activate the oocyte, undergo nuclear decondensation, form the male pronucleus, and then fuse with the female pronucleus. Finally, with the advent of intracytoplasmic sperm injection (ICSI), sperm function testing has assumed a role of even less importance. In recent years, ICSI has been utilized in greater than half of the ART cycles conducted each year. Many programs are applying 100% ICSI cycles [1]. As fertilization, blastocyst, and pregnancy rates improve in the contemporary ART lab, more and more logical questions are being asked about the proper role for sperm function testing. This chapter reviews techniques available for sperm evaluation and examines the issues surrounding their utilization in the modern ART program.

Patient history

A thorough history of the infertile couple at the time of the initial consultation will frequently reveal conditions that could affect semen quality. Some of the important factors to consider are as follows:

1. Reproductive history, including previous pregnancies with this and other partners.

2. Sexual interaction of the couple, including frequency and timing of intercourse along with the duration of their infertility.
3. Past medical and surgical history: specific attention should be paid to sexually transmitted diseases, prostatitis, or epididymitis, along with scrotal trauma or surgery—including varicocele repair, vasectomy, inguinal herniorrhaphy, and vasovasostomy.
4. Exposure to medication, drugs, toxins, and adverse environmental conditions such as temperature extremes in occupational and leisure activities, either in the past or in the present.

Semen analysis

The hallmark of the evaluation of the male remains the diagnostic semen analysis. It is well-known that the intra-patient variability of semen specimens from fertile men can be significant over time [2]. This variability decreases the diagnostic information that can be obtained from a single analysis, often necessitating additional analyses. What is also apparent from literature that analyses samples from “infertile” patients is that the deficiencies revealed may not be sufficient to prevent pregnancy from occurring. Rather, they may simply lower the probability of pregnancy, resulting in so-called subfertility. Clearly, the overall prognosis for a successful pregnancy is dependent on the complex combination of variables in semen quality coupled with the multiple factors inherent in the female reproductive system that must each function flawlessly. The commonly accepted standard for defining the normal semen analysis is the criteria defined by the World Health Organization (WHO). These parameters for the fourth, fifth, and most recent sixth edition are listed in [Table 6.1](#).

The normal or reference values for semen analyses have been altered with each new edition of WHO-defined criteria. The values from the current (sixth) edition have been derived from a retrospective look at the semen parameters of men with two to seven days of abstinence whose partner conceived within 12 months after the cessation of the use of contraception [3, 4]. There are significant changes in the parameters listed in the fifth and sixth edition compared with past editions. Some of these changes are due to observations made of the semen samples from the patients just mentioned. These real differences in declining sperm concentrations, motility, and normal morphology are thought to be due to environmental influences. However, the drastic changes in the morphology reference values are primarily due to the suggested use of the Kruger strict morphology method in the fifth and sixth edition. The value of this method will be discussed in the sperm morphology section of this chapter.

Collection of the specimen

When the semen analysis is scheduled, instructions must be given to the patient to ensure the collection of an optimum semen sample. Written instructions are useful, especially if the patient

TABLE 6.1 World Health Organization Reference Values for Semen Analysis

Parameter	Reference Values		
	Fourth Edition	Fifth Edition	Sixth Edition
Volume	>2.0 mL	1.5 (1.4–1.7)	1.4 (1.3–1.5)
Sperm concentration	20×10^6	$15 (12\text{--}16) \times 10^6$	$16 (15\text{--}18) \times 10^6$
Total sperm count	40×10^6	$39 (33\text{--}46) \times 10^6$	$39 (35\text{--}40) \times 10^6$
Total motility	50%	40% (38–42)	42% (40–43)
Progressive motility	25	32% (31–34)	30% (29–31)
Vitality	50%	58% (55–63)	54% (50–56)
pH	7.2	7.2	7.2
Morphology	15%	4% (3.0–4.0)	4% (3.9–4.0)

Source: Data from [5–7].

Note: Liquefaction: Complete within 60 minutes at room temperature (fifth edition) and at 37°C (sixth edition). Appearance: Homogeneous, grey, and opalescent. Consistency: Leaves pipette as discrete droplets. Leukocytes: Fewer than 1 million/mL.

is collecting the specimen outside of the clinical setting. During the initial infertility evaluation, a semen specimen should be obtained following a two- to seven-day abstinence from sexual activity [2]. A shorter period of time may adversely affect the semen volume and sperm concentration, although it may enhance sperm motility. A longer period of abstinence may reduce sperm motility. Considering the natural variability in semen quality that all men exhibit, the initial semen collection may not accurately reflect a typical ejaculate for that patient. A second collection, with a two-to seven-day abstinence period, can eliminate the tension associated with the initial semen collection, and provide a second specimen from which a typical set of semen parameters can be determined. An additional cause of variable semen quality can be the site of collection. Understandably, many men are inhibited by collecting their semen sample at the clinic. Although collecting at home is less intimidating, it is not always practical due to distance or schedules. In the case where the semen sample is collected at the clinic, the second and subsequent collections are usually better than the first due to an increase in the patient's comfort level. The second collection may also be used to determine the optimal abstinence period for a patient. Masturbation is the preferred method of collection. The use of lubricants is discouraged since most are spermicidal. However, some mineral oils and a few water-based lubricants are acceptable. Since masturbation may present significant difficulty for some men, either in the clinic or at home, an alternative method of collection must be available. The use of certain silastic condoms (seminal collection devices) during intercourse may be an acceptable second choice. Interrupted intercourse should not be considered, as this method tends to lose the sperm-rich initial few drops of semen while transferring many bacteria to the specimen container [2, 4].

Care of the specimen

Appropriate care of the ejaculate between collection and examination is important. Specimens should be collected only in approved, sterile, non-toxic, plastic, disposable cups. Many other plastic containers are toxic to sperm, especially if the sperm is allowed to remain in the containers for the duration of time that it takes to deliver the specimen from off-site. Washed containers

may contain soap or residue from previous contents, which can kill or contaminate the sperm. Delivery of the semen to the laboratory should occur within 60 minutes of collection, and the specimen should be kept at room temperature during transport. These recommendations are designed to maintain optimal sperm viability until the time of analysis.

Container labelling

The information recorded on the specimen container label should include the male's name along with a unique identifying number. Typically, a birth date, or a clinic-assigned patient number is used. Other helpful information recorded on the label should include the date and time of collection and the number of days since the last ejaculation. When the specimen is received from the patient, it is important to confirm that the information provided on the label is complete and accurate and documented accordingly.

Examination of the specimen

Liquefaction and viscosity

When the semen sample arrives in the laboratory, it is checked for liquefaction and viscosity. Although similar, these factors are distinct from each other [8, 9]. Liquefaction is a natural change in the consistency of semen from a semi-liquid to a liquid. Before this process is completed, sperm are contained in a gel-like matrix that prevents their homogeneous distribution. Aliquots taken from this uneven distribution of sperm for the purpose of determining concentration, motility, or morphology may not be truly representative of the entire specimen. As liquefaction occurs over 15–30 minutes, sperm are released and distributed throughout the semen. Incomplete liquefaction may adversely affect the accuracy of the semen analysis by preventing this even distribution of sperm within the sample. The coagulum that characterizes freshly ejaculated semen results from secretions from the seminal vesicles. The liquefaction of this coagulum is the result of enzymatic secretions from the prostate. Watery semen, in the absence of a coagulum, may indicate the absence of the ejaculatory duct or non-functional seminal vesicles. Inadequate liquefaction, in the presence of a coagulum, may indicate a deficiency of prostatic enzymes [10, 11].

Viscosity refers to the liquefied specimen's tendency to form drops from the tip of a pipette. If drops form and fall freely, the specimen has a normal viscosity. If drops will not form or the semen cannot be easily drawn up into a pipette, viscosity is high. This high viscosity remains, even after liquefaction has taken place. Highly viscous semen may also prevent the homogeneous distribution of sperm. Treatment with an enzyme, such as chymotrypsin [12], or aspiration of semen through an 18-gauge needle may reduce the viscosity and improve the distribution of sperm before an aliquot is removed for counting. Any addition of medium containing enzymes should be recorded, as this affects the actual sperm concentration. The new volume must be factored in when calculating the total sperm count.

Semen volume

Semen volume can be measured with a serological pipette that is graduated to 0.1 mL. The volume is recorded and multiplied by the sperm concentration to obtain the total count of sperm in the sample. A normal seminal volume before dilution is considered to be >1.3 mL [13].

Sperm concentration

A variety of counting chambers are available for determining sperm concentration. These include but are not limited to the haemocytometer, Makler counting chamber, and MicroCell. Regardless of the type of chamber used, an aliquot from a homogeneous, mixed semen sample is placed onto a 37°C chamber. The chamber is manufactured to a certain depth, which allows the sperm to distribute evenly in a very thin layer. Sperm within a grid are counted, and a calculation is made according to the formula for the type of chamber used. Accuracy is improved by including a greater number of rows, squares, or fields in the count. Sperm counts should be performed immediately after loading semen onto the chamber. As indicated earlier, a particular patient's sperm count may vary significantly from one ejaculate to another. This observation holds true for both fertile and infertile males, further complicating the definition of a normal range for sperm concentration. Demographic studies employing historic controls were used to define a sperm concentration of <16 million/mL as abnormal (sixth edition; [13]). Several investigators had observed that significantly fewer pregnancies occurred when men had sperm counts <16 million/mL; however, the prognosis for pregnancy did not increase proportionately with sperm concentrations above this threshold.

Sperm motility

Sperm motility may be affected by many factors:

- Patient's age and general health
- Length of time since the last ejaculation (abstinence)
- Patient's exposure to outside influences such as excessive heat or toxins
- Method of collection
- Length of time and adequacy of handling from collection to analysis

When the aliquot of semen is placed on the 37°C counting chamber, the count and motility should be determined immediately. If a chamber with a grid is used to count the sperm, the motility can be determined at the same time as the concentration by using a multiple-click cell counter to tally motile and

non-motile sperm and then totalling these numbers to arrive at the true sperm concentration. The accuracy of the concentration and of the motility improves as more sperm are counted. If a wet-mount slide is used to determine motility, more than one area of the slide should be used, and each count should include at least 200 sperm. Prior to examining the specimen for motility, the slide or counting chamber should be examined for signs of sperm clumping. Agglutination refers to motile sperm sticking to other sperm. This can be head-to-head, head-to-tail, or tail-to-tail. This may indicate the presence of sperm antibodies in the semen. The severity of sperm agglutination is evaluated on a scale of 1 to 5. A score of 1 is isolated (<10 sperm/agglutinate); 2 is moderate (10–50 sperm/agglutinate); 3 is large (>50 sperm/agglutinate); and 4 is gross (all sperm agglutinated). The type and degree of agglutination should be recorded. This should not be confused with clumping of sperm to other cellular debris in the semen, or non-motile sperm stuck to each other (aggregation). In any case, sperm clumping may affect the accuracy of both the sperm count and the motility [1, 13].

Motility is one of the most important prerequisites for achieving fertilization and pregnancy. The head of the sperm must be delivered a great distance *in vivo* through the barriers of the reproductive tract to the site of the oocyte. Sperm must have sufficient motility to penetrate both the layers of corona cells and the zona pellucida before fusing with the oocyte cell membrane (oolemma). An exact threshold level of motility that is required to accomplish fertilization and pregnancy, however, has never been described [12]. This may be due to variables in the equipment and techniques used in assessing motility.

Progression

While sperm motility represents the quantitative parameter of sperm movement expressed as a percentage, sperm progression represents the quality of sperm movement expressed on a subjective scale. A typical scale attempts to depict the type of movement exhibited by most of the sperm visualized on a chamber grid. Progression of sperm may also be calculated with sperm motility as a percentage of sperm exhibiting "progressive motility." With the advent of successful micro-assisted fertilization, progression has assumed more limited utility. Nevertheless, for those laboratories that quantify progression of motility separately, a score of 0 means no motility; 1 means motility with vibratory motion without forward progression; 2 means motility with slow, erratic forward progression; 3 means motility with relatively straightforward motion; and 4 is motility with rapid forward progression [13].

Sperm vitality

When a motility evaluation yields a low proportion of moving sperm (less than 50%), a vitality stain may be beneficial. This is a method used to distinguish non-motile sperm that are living from those that are dead. This technique will be discussed later in the sperm function section.

Additional cell types

While observing sperm in a counting chamber or on a slide, additional cell types may also be seen. These include endothelial cells from the urethra, epithelial cells from the skin, immature sperm cells, and white blood cells. The most common and significant of these cell types is referred to collectively as "round cells." These include immature sperm cells and white blood cells. In order to distinguish between them, an aliquot of semen can be placed in a

thin layer on a slide and air-dried. The cells are fixed to the slide and stained using a Wright–Giems or Bryan–Leishman stain. When viewed under 400 \times or 1000 \times power, cell types may be differentiated primarily by their nuclear morphology. Immature sperm have one to three round nuclei within a common cytoplasm. Polymorphonuclear leukocytes may also be multinucleate, but the staining method will typically reveal characteristic nuclear bridges between their irregularly shaped nuclei [1]. A peroxidase stain may be used to identify granulocytes and to differentiate them from the immature sperm. The presence of greater than one million white blood cells per one millilitre of semen may indicate an infection in the urethra or accessory glands, which provide the majority of the seminal plasma. Such infections could contribute to infertility [1, 14]. These samples can be cultured so that the offending organism can be identified, and appropriate treatment can be instituted. Besides bacteria, white blood cells on their own can contribute to infertility. They can especially be a detrimental factor in the *in vitro* fertilization (IVF) process. The white blood cells can be removed by centrifugation of the semen sample through a layer of silica beads; the toxins produced by the cells, called leukokines, may pass through the layer and concentrate in the medium below containing the sperm. If the sperm is to be used in the insemination of oocytes, the concentrated toxins will be in contact with the oocytes for several hours. These toxins may cause detrimental effects to the oocytes and to the embryos that develop from fertilization. The detrimental effects of white blood cells in a semen sample can be ameliorated by the application of ICSI, which eliminates the long-term exposure of oocytes to toxins.

Sperm morphology

Sperm morphology should be assessed using Kruger strict criteria according to WHO sixth edition standards (Figure 6.1) [15]. It is recommended that the slide be stained with Papanicolaou staining, although other methods can be utilized with proper evaluation and validation [13]. At least 200 sperm must be counted using bright field optics at $\times 1000$ magnification with oil immersion [13]. WHO sixth edition criteria for assessing normal forms include the following:

- Head: Smooth; oval configuration; length, 5–6 μm , diameter 2.5–3.5 μm ; acrosome, must constitute 40%–70% of the sperm head.
- Mid-piece: Slender, axially attached; <1 μm in width and approximately 1.5 μm in head length; no cytoplasmic droplets, >50% of the size of the sperm head.
- Tail: Single, unbroken, straight, without kinks or coils, approximately 45 μm in length (Figure 6.2) [13, 15–17].

As described by Kruger et al., sperm forms that are not clearly normal should be considered abnormal. The presence of 4% or greater normal sperm morphology should be interpreted as a normal result. Normal morphology of <4% is abnormal [16, 17]. Normal sperm morphology has been reported to be directly related to fertilization potential. This may be due to the inability of abnormal sperm to deliver genetic material to the cytoplasm of the egg. From video recordings, it appears that abnormal sperm are more likely to have diminished, aberrant, or absent motility. This reduced or unusual motility may result from hydrodynamic inefficiency due to the head shape, abnormalities in the tail structure that prevent normal motion, and/or

deficiencies in energy production necessary for motility [18, 19]. In addition to compromised motility, abnormal sperm do not appear to bind to the zona of the egg as well as normal sperm. This has been demonstrated in studies employing the hemizona binding assay [20]. IVF has helped to further elucidate the role that normal sperm morphology plays in the fertilization process and in pregnancy.

Computer-assisted semen analysis

Computer-assisted semen analysis (CASA) was initially developed to improve the accuracy of manual subjective semen analysis. Its goal is to establish a standardized, objective, reproducible test for sperm concentration, motility, and morphology. This technique also characterizes sperm movement. The automated sperm movement measurements—known as kinematics—include straight-line velocity, curvilinear velocity, and mean angular displacement (Table 6.2). The use of CASA requires specialized equipment, including a phase contrast microscope, video camera, video recorder, video monitor, computer, and printer.

To perform CASA, sperm are placed on a chamber or in a capillary specific to the CASA device and then viewed under an internal magnification device. The video camera records the moving images of the sperm cells, and the computer digitizes these frames accordingly. The digitized images consist of pixels whose changing locations are recorded frame by frame. A total of 30–200 frames per minute are produced. The changing locations of each sperm are recorded, and their trajectories are computed (Figure 6.3) [21]. In this manner, hyperactive motion can also be detected and recorded. Hyperactive sperm exhibit a whip-like, thrashing movement, which is thought to be associated with sperm that are removed from seminal plasma and ready to fertilize the oocytes [21, 22]. Historically, the validity and reproducibility of results have kept CASA from becoming a standard procedure in the andrology laboratory. However, with advances in technology these devices have become more accurate, allowing for an opportunity for a more standardized and objective semen analysis within the field [23]. The accuracy of sperm concentration appears to be diminished in the presence of either severe oligospermia or excessive numbers of sperm. In cases of oligospermia, counts may be overestimated due to the machine counting debris as sperm. High concentrations of sperm may be underestimated in the presence of clumping. High sperm concentrations can also cause overestimations in counting due to the way the software handles collisions between motile sperm and non-motile sperm. In these cases, diluting the sample may improve the accuracy of the count [22, 23]. Sperm motion parameters identified by CASA have been assessed by several investigators for their ability to predict fertilization potential. Certain types of motion have been determined to be important in achieving specific actions related to fertilization, such as cervical mucus penetration and zona binding. However, the overall potential of CASA for predicting pregnancy remains to be elucidated. Persistent questions about the reproducibility and reliability of results and their interpretation continue to limit the routine use of CASA. The use of fluorescent DNA staining with CASA may help to improve its reliability. In addition, as the kinematics of sperm motion becomes better understood, CASA may play an integral role in determining the optimal method of assisted reproductive technique that should be utilized for specific types of male factor patients [24].

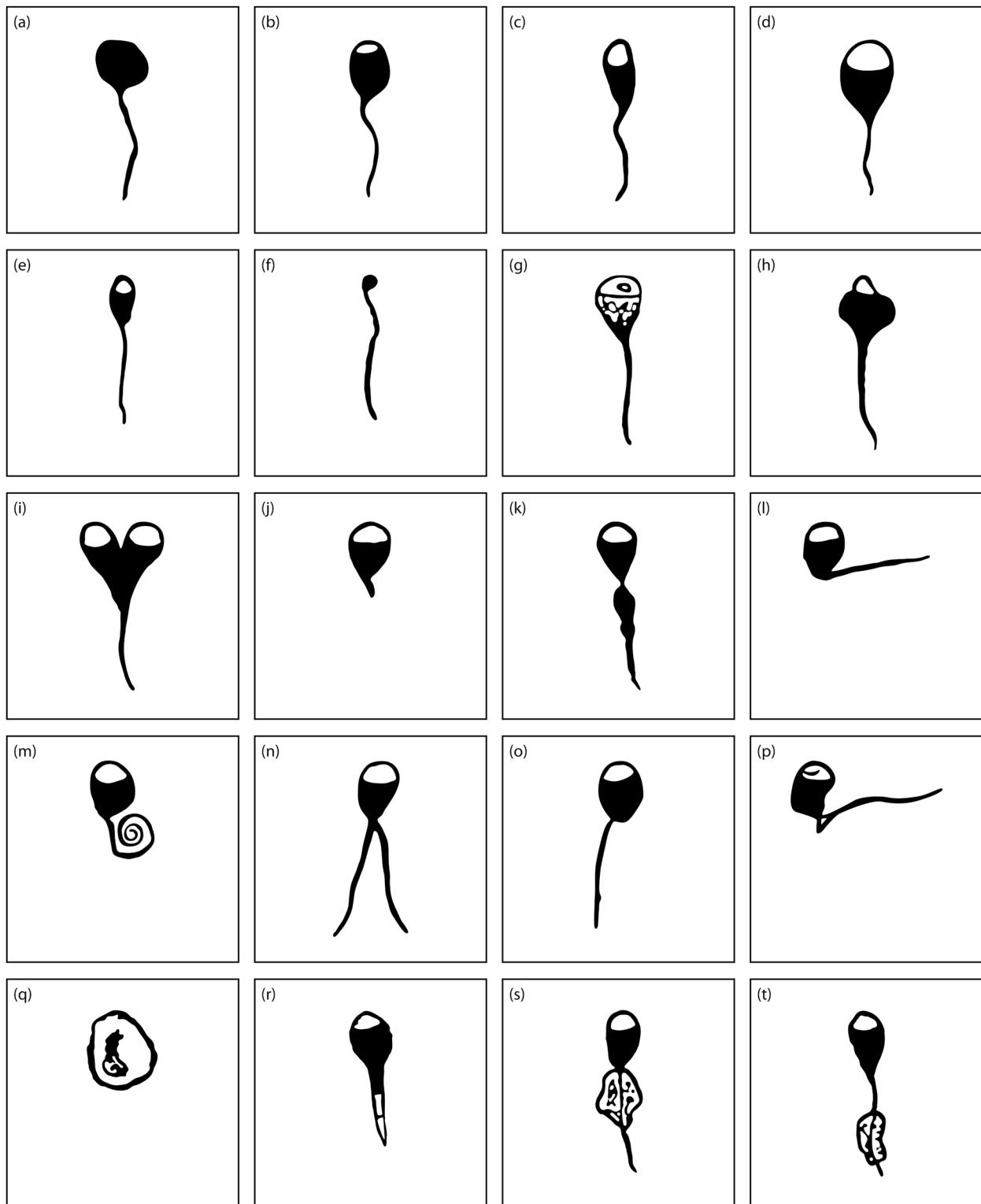


FIGURE 6.1 Different types of sperm malformations. (a) Round head/no acrosome; (b) small acrosome; (c) elongated head; (d) megaloh head; (e) small head; (f) pinhead; (g) vacuolated head; (h) amorphous head; (i) bicephalic; (j) loose head; (k) amorphous head; (l) broken neck; (m) coiled tail; (n) double tail; (o) abaxial tail attachment; (p) multiple defects; (q) immature germ cell; (r) elongated spermatid; (s) proximal cytoplasmic droplet; and (t) distal cytoplasmic droplet. (From [17], with permission.)

TABLE 6.2 Kinematic Measurements in Computer-Assisted Semen Analysis

Symbol	Name	Definition
VSL	Straight-line velocity	Time average velocity of the sperm head along a straight line from its first position to its last position
VCL	Curvilinear velocity	Time average velocity of the sperm head along its actual trajectory
VAP	Average path velocity	Time average velocity of the sperm head along its average trajectory
LIN	Linearity	Linearity of the curvilinear trajectory (VSL/VCL)
WOB	Wobble	Degree of oscillation of the actual sperm head trajectory around its average path (VAP/VCL)
STR	Straightness	Straightness of the average path (VSL/VAP)
ALH	Amplitude of lateral head	Amplitude of variations of the actual sperm head trajectory about its average trajectory displacement (the average trajectory is computed using a rectangular running average)
RIS	Riser displacement	Point-to-point distance of the actual sperm head trajectory to its average path (the average path is computed using an adaptive smoothing algorithm)
BCF	Beat-cross frequency	Time average rate at which the actual sperm trajectory crosses the average path trajectory
HAR	Frequency of the fundamental	Fundamental frequency of the oscillation of the curvilinear trajectory around its average harmonic path (HAR is computed using the Fourier transformation)
MAG	Magnitude of the amplitude	Squared height of the HAR spectral peak (MAG is a measure of the peak to fundamental harmonic peak dispersion of the raw trajectory about its average path at the fundamental frequency)
VOL	Area of fundamental harmonic	Area under the fundamental harmonic peak in the magnitude spectrum (VOL is a harmonic measure of the power-bandwidth of the signal)
CON	Specimen concentration	Concentration of sperm cells in a sample in millions of sperm per mL of plasma or medium
MOT	Percentage motility	Percentage of sperm cells in a suspension that are motile (in manual analysis, motility is defined by a moving flagellum; in computer-assisted semen analysis, motility is defined by a minimum VSL for each sperm)

Source: Data from [22].

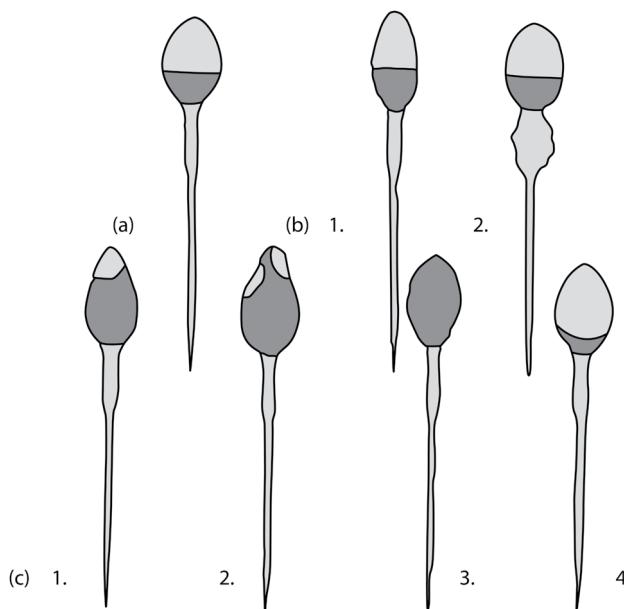


FIGURE 6.2 A diagrammatic representation of quick-stained spermatozoa. (a) Normal form; (b.1) slightly amorphous head; (b.2) neck defect; (c.1 and 2) abnormally small acrosome; (c.3) no acrosome; and (c.4) acrosome 70% of sperm head. (From [17], with permission.)

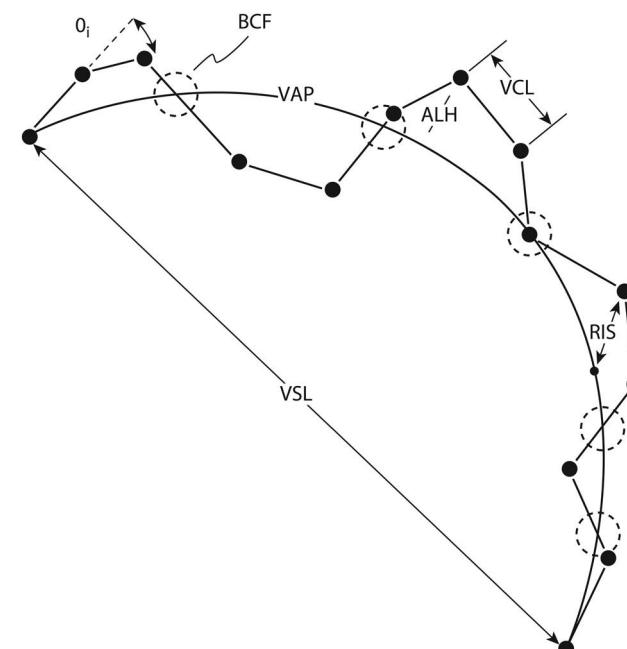


FIGURE 6.3 Examples of kinematic measurements involved in a single-sperm tracing.

Abbreviations: ALH, amplitude of lateral head; BCF, beat-cross frequency; RIS, riser displacement; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity. (Compiled from data in [22].)

Sperm antibodies

Because mature sperm are formed after puberty, they can be recognized as foreign protein by the male immune system. In the testes, the sperm are protected from circulating immunoglobulins by the tight junctions of the Sertoli cells. As long as the sperm are contained within the lumen of the male reproductive tract, they are sequestered and protected from the immune system, and no antibodies form to their surface antigens. If there is a breach in this so-called blood–testis barrier, an immune response may be initiated. The most common causes of a breach in the reproductive tract, which could initiate antibody formation, include vasectomy, varicocele repair, testicular biopsy, torsion, trauma, and infection [25, 26]. Once formed, antibodies are secreted into the fluids of the accessory glands, specifically the prostate and seminal vesicles. At the time of ejaculation, the fluids from these glands contribute most of the volume to the seminal plasma. These antibodies can then come into contact with the sperm and may cause them to clump. In women, the atraumatic introduction of sperm into the reproductive tract as a result of intercourse or artificial insemination does not appear to be a factor in the production of sperm antibodies. However, events that induce trauma or introduce sperm to the mucous membranes outside of the reproductive tract can induce antibody formation. Proposed examples of such events include trauma to the vaginal mucosa during intercourse or the deposition of sperm into the gastrointestinal tract by way of oral or anal intercourse [26]. There are several tests currently employed for detecting the presence of sperm antibodies. The two most common are the mixed agglutination reaction (MAR) and the immunobead binding test. The widespread application of ICSI has reduced the use and benefit of these tests and is commonly now used to circumvent any antibody formation.

The MAR

This test is performed by mixing semen, IgG- or IgA-coated latex beads or red blood cells, and IgG or IgA antiserum on a microscope slide. The slides are incubated and observed at 400 \times magnification. At least 200 sperm are counted. If antibodies are present, the sperm will form clumps with the coated latex beads or coated red blood cells. If antibodies are absent, the sperm will swim freely. The level of antibody concentration considered to be clinically relevant must be established by each centre conducting the test. The WHO considers a level of binding of $\geq 50\%$ to be clinically significant. This test is used only for detection of direct antibodies in men, and is not specific for the location of bead attachment to the sperm.

The immunobead binding test

The direct immunobead test is performed by combining IgG- or IgA-coated latex beads and washed sperm on a slide. After washing, the sperm is placed on a slide with IgG- or IgA-coated latex beads and is read at 200 \times or 400 \times magnification. Similar to the MAR test, this test can be used for the detection of direct antibodies in men. If antibodies are absent, the beads will not attach to the sperm. If antibodies are present, the small beads will attach directly to the sperm. This test provides potentially greater information than the MAR, as results consider the number of sperm bound by beads, the type of antigen involved in binding, and the specific location where the bead is bound to the sperm.

This test may also be used to detect antibodies produced in a woman's serum, follicular fluid, or cervical mucus by incubating

these bodily fluids with washed sperm that have previously tested negative for antibodies. To perform an indirect test, known direct antibody-negative sperm are washed and incubated for one hour at 37°C with the bodily fluid to be tested. The sperm are then washed free of the bodily fluid, resuspended, and mixed on a slide with IgG- or IgA-coated latex beads. The test is interpreted by noting the percentage and location of the bead attachment. Historically, the third edition WHO standard considered the level of binding of $\geq 20\%$ as representing a positive test. The fourth, fifth, and sixth edition WHO standard considers a level of $\geq 50\%$ to be a positive test. The level of binding of $\geq 50\%$ is commonly considered to be clinically significant [12, 27]. The clinical value of anti-sperm antibody testing is predicated on the observation that the presence of a significant concentration of antibodies may impair fertilization. It has been reported that antibody-positive sperm may have difficulty penetrating cervical mucus. Although in these cases intrauterine insemination or IVF may improve the prognosis for fertilization, antibody levels $>80\%$, coupled with subpar concentration, motility, or morphology, may necessitate the addition of ICSI in order to achieve the highest percentage of fertilization [24].

Sperm vitality

An intact plasma membrane is an integral component of, and possibly a biologic/diagnostic indicator for, sperm viability. The underlying principle is that viable sperm contain intact plasma membranes that prevent the passage of certain stains, whereas nonviable sperm have defects within their membranes that allow for staining of the sperm. Several so-called vital stains have been employed for this purpose. They include eosin Y, trypan blue, and/or nigrosin [28]. When viewed with either bright field or phase contrast microscopy, these stains allow for the differentiation of viable, non-motile sperm from dead sperm. This procedure may, therefore, play a significant role in determining the percentage of immotile sperm that are viable and available for ICSI. Unfortunately, dyes such as eosin Y are specific DNA probes that may have toxic effects if they enter a viable sperm or oocyte, which precludes the use of these sperm that have been exposed to the dyes for ICSI or insemination. Flow cytometry has also been utilized for the determination of sperm viability. Like vital staining, flow cytometry is based on the principle that an intact plasma membrane will prevent the passage of nucleic acid-specific stains. Some techniques, such as the one described by Noiles et al., employ dual staining, which can differentiate between an intact membrane and a damaged membrane [29].

Hypo-osmotic swelling test

Another means of assessing the sperm plasma membrane is the hypo-osmotic swelling test (HOST). This assay is predicated upon the observation that all living cells are permeable to water, although to different degrees. The human sperm membrane has one of the highest hydraulic conductivity coefficients (2.4 $\mu\text{L}/\text{min}/\text{atm}$ at 22°C) of any mammalian cell [30].

As originally described, the HOST involves placing a sperm specimen into hypotonic conditions of approximately 150 mOsmol [31]. This environment, while not sufficiently hypotonic to cause cell lysis, will cause swelling of the sperm cells. As the tail swells, fibres cause the sperm tail to curl, and this change can be detected by phase contrast microscopy, differential interference contrast, or Hoffman optics. The normal range for a positive

test is typically considered to be a score $\geq 60\%$; that is, at least 60% of the cells demonstrate curling of the tails. A negative test is defined as $<50\%$ curling [32]. This test generated a significant amount of initial interest, and several investigators compared it to the sperm penetration assay (SPA) as an *in vitro* surrogate for fertilization, reporting good correlation [33, 34]. The use of the HOST is not increasing significantly, but can be beneficial in specific cases, such as those with nonmotile sperm, for example patients with ciliary dyskinesia [35].

Assays of the sperm acrosome

The acrosome is an intracellular organelle, similar to a lysosome, which forms a cap-like structure over the apical portion of the sperm nucleus [36]. The acrosome contains multiple hydrolytic enzymes, including hyaluronidase, neuraminidase, proacrosin, phospholipase, and acid phosphatase, which, when released, are thought to facilitate sperm passage through the cumulus mass, and possibly the zona pellucida as well (Figure 6.4). In fact, only acrosome-reacted sperm is capable of penetrating the zona pellucida, binding to the oolemma, and fusing with the oocyte [37]. Once sperm undergoes capacitation, it is capable of an acrosome reaction. This reaction is apparently triggered by the fusion of the sperm plasma membrane with the outer acrosomal membrane at multiple sites, leading to the diffusion of the acrosomal enzymes into the extracellular space. This leads to the dissolution of the plasma membrane and acrosome, leaving the inner acrosomal membrane exposed over the head of the sperm (Figure 6.5). Although electron microscopy has produced many elegant pictures of acrosome-intact and acrosome-reacted sperm, it is not always possible to know whether sperm that fail to exhibit an acrosome have truly acrosome reacted, or could possibly be dead. In addition, electron microscopy is not a technique that is available to all andrologists.

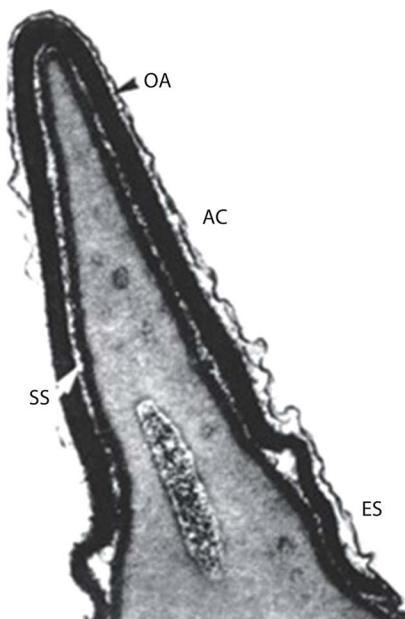


FIGURE 6.4 Sperm head with intact acrosome.

Abbreviations: AC, acrosomal cap; ES, equatorial segment; OA, outer acrosomal membrane; SS, sub-acrosomal space. (From [17], with permission.)

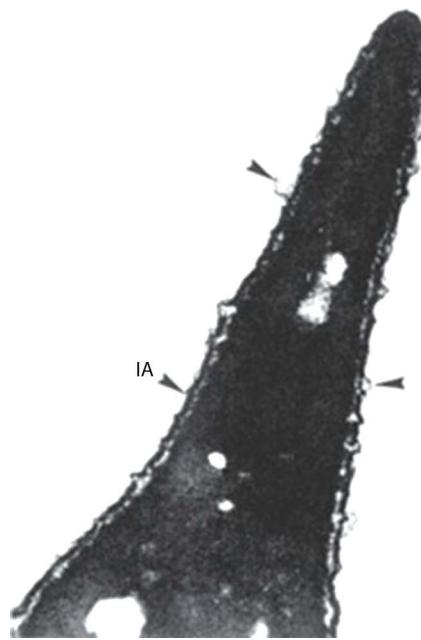


FIGURE 6.5 Acrosome-reacted sperm.

Abbreviation: IA, inner acrosomal membrane. (From [17], with permission.)

This has led to the necessity for the development of biochemical markers for the acrosome reaction. Throughout the 1970s, 1980s, and 1990s, multiple biochemical tests were described using a variety of lectins, antibodies, and stains. Although they apparently correlated well with electron microscopy, the tests were still time-consuming and difficult to perform [35, 38]. Contemporary assays for the determination of acrosomal status employ fluorescent plant lectins or monoclonal antibodies, which can be detected much more easily with fluorescence microscopy [39, 40]. This test is not routinely used, as the utilization of ICSI circumvents the need for acrosome binding to the zona pellucida.

Other biochemical tests

As noted earlier, one of the predominant enzymes that is present in the acrosome is proacrosin. The enzymatic action of acrosin is not necessarily correlated to the presence of an intact acrosome; therefore, assays for the presence of acrosin have been described [41]. Acrosin activity has been reported to be greater in fertile men than in infertile men [42]; however, there are no prospective evaluations correlating acrosin activity to fertilization rates in ART patients. Like all other tissues that require energy synthesis and transport, spermatozoa contain measurable levels of creatinine phosphokinase. Two isomers, CK-M and CK-B, have been described, and differences have been noted in the levels of these isomers in semen specimens from fertile and infertile men. Specifically, CK-M levels exceed CK-B levels in normospermic males, while CK-B levels are greater in spermatozoa from oligospermic males [43]. In this same study, researchers found that semen samples in which CK-M/CK-B ratios exceeded 10% exhibited higher fertilization rates in IVF than specimens with lower ratios. Few other studies have addressed this topic.

Sperm penetration assay

The SPA or hamster egg penetration assay was initially described by Yanagimachi et al. in 1976 [44]. It measures the ability of sperm to undergo capacitation and the acrosome reaction, penetrate the oolemma, and then decondense. In this test, oocytes from the golden hamster are first treated in order to remove the zona pellucida. As one of the functions of the zona is to confer species specificity, its presence would preclude performance of this test. However, zona removal obviously prohibits the SPA from being able to assess sperm for the presence of zona receptors.

Following zona removal, human sperm are incubated for 48 hours along with hamster oocytes, and the number of penetrations with nuclear decondensation is calculated. As originally described, it was hoped that the test would correlate with the ability of human sperm to fertilize human oocytes *in vitro*. Although the test was designed to assess the ability of sperm to fuse to the oolemma, it also indirectly assesses sperm capacitation, the acrosome reaction, and the ability of the sperm to be incorporated into the ooplasm. Unfortunately, however, intrinsic in the design of the test is its inability to assess the sperm's ability to bind to—and penetrate through—the zona pellucida. This factor continues to be one of the major criticisms that plague this test. Throughout the 1980s, multiple modifications of the SPA were published. These included modifications of the techniques for sperm preparation prior to the performance of the assay, such as inducing the acrosome reaction or incubation with TEST yolk buffer (Fuji Film Irvine Scientific, Irvine, CA), changes in the protocol methodology itself, and modifications of the scoring system [45, 46]. Published reports demonstrated widely varying conclusions, such as the finding that the SPA could identify anywhere from 0% to 78% of men whose sperm would fail to fertilize oocytes in ART procedures [47]. Most criticisms of the SPA literature centre on the poor standardization of the assay, the poor reproducibility of the test, and the lack of a standard normal range.

Although some reports suggest a correlation between the SPA and fertility, neither a large literature review [47] nor a prospective long-term (five-year) follow-up study demonstrated such a correlation [48]. In fact, a meta-analysis of 2906 subjects from 34 prospective, controlled studies suggested that the SPA is a poor predictor of fertilization [49]. In light of these considerations, support for this test has gradually waned.

Hemizona assay

Research has demonstrated a significant correlation between tests of sperm–zona pellucida binding and subsequent fertilization in ART. This led the European Society for Human Reproduction and Embryology (ESHRE) Andrology Special Interest Group to recommend inclusion of such tests in the advanced evaluation of the male [50]. Like the SPA, the hemizona assay (HZA) employs sperm and nonviable oocytes in an *in vitro* assessment of fertilization [51]. In this test, however, both gametes are human in origin. As described, the HZA assesses the ability of sperm to undergo capacitation, acrosome react, and bind tightly to the zona. Classically, oocytes that failed to fertilize during an ART procedure are bisected, and then sperm from a proven fertile donor (500,000/mL) is added to one hemizona, while sperm from the subject male is added to the other hemizona. Following a four-hour incubation, each hemizona is removed and pipetted in order to dislodge loosely attached sperm. A comparison or hemizona index (HZI) is then calculated by dividing the number

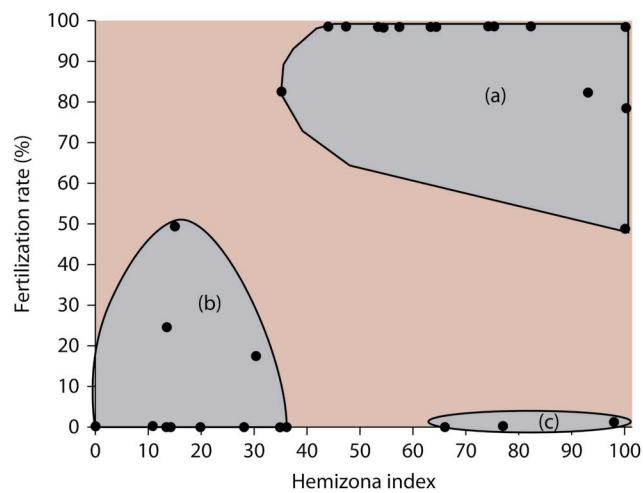


FIGURE 6.6 Cluster analysis of hemizona assay index and fertilization rate. (a) Good fertilization; (b) poor fertilization; and (c) false-positive hemizona assay index. (From [55], with permission.)

of test sperm tightly bound to the hemizona by the number of control (fertile) sperm bound to the other hemizona:

$$\text{HZI} = \frac{\text{Number of test sperm bound}}{\text{Number of control sperm bound} \times 100}$$

This test assesses the ability of sperm to bind to the zona itself. Although the HZA is relatively expensive, labour intensive, and difficult to perform, there are some data that suggest that the HZA may help to identify individuals with a poor prognosis for success with ART (Figure 6.6) [52, 53]. A more recent prospective study employing receiver operating characteristic curve analysis has also suggested that HZA results may be used to predict subsequent fertilization in ART procedures with both high sensitivity and specificity [54]. Unlike several other tests of sperm function, a cut-off value (35%) has been identified as a predictor of IVF success. In addition, pregnancy rates in patients with values over 30 have been shown to be significantly higher than those in patients with values under 30 (40.6% vs. 11.1%, $p < 0.05$) [55]. The use of ICSI provides an alternative for successful fertilization in the event that a sperm to zona binding issue exists.

Mannose binding assay

Another historical test, the mannose binding assay, was used to assess the ability of sperm to bind to the zona. This *in vitro* procedure is based on a series of observations that suggest that sperm–oocyte interaction involves the recognition by a sperm surface receptor of a specific complementary receptor on the surface of the zona pellucida. This zona receptor appears to be a glycoprotein, the predominant sugar moiety of which is mannose [56]. In an elegant series of experiments, Mori et al. determined that sperm–zona binding could be curtailed by the addition of a series of sugars to the incubating media. Although many sugars impaired binding, the addition of mannose totally inhibited sperm–oocyte interaction [57]. *In vitro* assays in which labelled probes of mannose conjugated to albumin are co-incubated with semen specimens allow for the differential staining



FIGURE 6.7 Mannose-positive (brown) and mannose-negative (clear) sperm. (Courtesy of Tammy Dey and Kaylen Silverberg.)

of sperm (Figure 6.7). Those that bind the probe are thought to possess the sperm surface receptor for the mannose-rich zona glycoprotein. Several investigators, including our group, have subsequently demonstrated that sperm from fertile populations exhibit greater mannose binding than do sperm from infertile males [58–60]. The application of ICSI has made this test obsolete.

Assays of sperm DNA integrity

The most current area of investigation into sperm function involves the assessment of sperm DNA integrity. Sperm chromatin has been demonstrated to be packaged very differently from chromatin in somatic cells. Specifically, the DNA is organized in such a manner that it remains very compact and stable [61]. As there are many ways in which this DNA organization or the sperm chromatin itself can be damaged, several assays of sperm chromatin assessment have been developed. There are two basic types of assays: direct assays, such as the “Comet” and “Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)” assays; and indirect assays, such as the sperm chromatin structure assay or acridine orange assay [62]. The direct assays detect actual breakages in the DNA, while the indirect assays measure the relative proportions of single-stranded (abnormal) and double-stranded (normal) DNA within the sperm following acid treatment. Data from several studies suggest that infertile men have a significantly greater amount of DNA damage than fertile men [61, 63, 64]. There is also a suggestion that this finding is similarly present in the male partners of couples experiencing recurrent miscarriage. Despite these reports, at the present time, there is no conclusive correlation between the results of sperm DNA integrity testing and pregnancy rates achieved either naturally or with the ARTs. As such, the Practice Committee of the American Society for Reproductive Medicine recommended that the routine testing of sperm DNA integrity should not be included in the evaluation of infertile couples [65].

Conclusion

In summary, there have been many recent advances in the diagnostic evaluation of sperm and sperm function. Although many tests of sperm function have been described, there remains a lack

of consensus as to the role of testing and the identification of the appropriate test(s) to perform. Owing to the complicated nature of sperm function, it is improbable that a single test will emerge with sufficient sensitivity, specificity, and positive and negative predictive values required of a first-line diagnostic tool for all affected men. A more likely scenario will be similar to that in female infertility, where a battery of tests—each evaluating a specific function—are employed as needed. Considering profound recent advances in gamete micromanipulation (e.g., ICSI), a more germane issue might be the overall relevance of sperm function testing in the contemporary andrology laboratory. Although this issue is quite controversial, it is likely that sperm function testing will continue to play a role in the evaluation of the infertile male. Just as ART is not the treatment of choice for all infertile women, it is not likely that micromanipulation will be the standard treatment for all infertile men. The gold standard of sperm function remains the ability to fertilize an oocyte *in vitro*. Therefore, in order to continue to address the preceding questions, it is incumbent upon investigators to design appropriate prospective trials to assess these tests thoroughly. Those tests that demonstrate a statistically significant correlation with fertilization *in vitro* must then undergo additional evaluation in order to assess clinical significance if we hope to develop an appropriate diagnostic algorithm.

References

1. Centers for Disease Control and Prevention. Assisted Reproductive Technology National Summary Report 2016. Available online: <https://www.cdc.gov/art/pdf/2016-report/ART-2016-National-Summary-Report.pdf> (accessed on 8 September 2020).
2. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 4th ed. New York, NY: Cambridge University Press, pp. 60–1, 1999.
3. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen, 5th ed. Geneva: WHO Press, pp. 223–5, 2010.
4. Cooper TG, Noonan E, von Eckardstein S, et al. World Health Organization reference values for human semen characteristics. Hum Reprod Update. 2010;16:231–45.
5. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 4th ed. New York, NY: Cambridge University Press, 1999, pp. 60–1.
6. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen, 5th ed. Geneva: WHO Press, 2010, pp. 223–5.
7. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen, 6th ed. Geneva: WHO Press, 2021, 9–82.
8. Alexander NJ. Male evaluation and semen analysis. Clin Obstet Gynecol. 1982;25:463–82.
9. Overstreet JW, Katz DF, Hanson FW, Foseca JR. A simple inexpensive method for objective assessment of human sperm movement characteristics. Fertil Steril. 1979;31:162–72.
10. Overstreet JW, Davis RO, Katz DF, Overstreet JW (eds.). Infertility and Reproductive Medicine. Clinics of North America. Philadelphia, PA: WB Saunders, pp. 329–40, 1992.
11. Koren E, Lukac J. Mechanism of liquefaction of the human ejaculate: I. Changes of the ejaculate proteins. J Reprod Fertil. 1979;56:493–500.
12. Lukac J, Koren E. Mechanism of liquefaction of the human ejaculate: II. Role of collagenase like peptidase and seminal proteinase. J Reprod Fertil. 1979;56:501–10.

13. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen, 6th ed. Geneva: WHO Press, pp. 1–159, 2021.
14. Cohen J, Aafjes JH. Proteolytic enzymes stimulate human spermatozoal motility and *in vitro* hamster egg penetration. *Life Sci.* 1982;30:899–904.
15. Van Voorhis BJ, Sparks A. Semen analysis: What tests are clinically useful? *Clin Obstet Gynecol.* 1999;42:957–71.
16. Gangi CR, Nagler HM. Clinical evaluation of the subfertile man. In: Infertility and Reproductive Medicine. Clinics of North America, Diamond MP, DeCherney AH, Overstreet JW (eds.). Philadelphia, PA: WB Saunders, pp. 299–318, 1992.
17. Sathananthan AH, Selvaraj K (eds.). Visual Atlas of Human Sperm Structure and Function for Assisted Reproductive Technique. Singapore: Serono, 1996.
18. Kruger TF, Acosta AA, Simmons KF, et al. Predictive value of abnormal sperm morphology in *in vitro* fertilization. *Fertil Steril.* 1988;49:112–17.
19. Kruger TF, Menkveld R, Stander FS, et al. Sperm morphologic features as a prognostic factor in *in vitro* fertilization. *Fertil Steril.* 1986;46:1118–23.
20. Katz DF, Overstreet JW. Sperm motility assessment by video micrography. *Fertil Steril.* 1981;35:188–93.
21. Coetzee K, Kruger TF, Lombard CJ. Predictive value of normal sperm morphology: A structured literature review. *Hum Reprod Update.* 1988;4:73–82.
22. Enginsu MF, Pieters MGEC, Dumoulin JCM, et al. Male factor as determinant of *in vitro* fertilization outcome. *Hum Reprod.* 1992;7:1136–40.
23. Tomlinson MJ, Pooley K, Simpson T, et al. Validation of a novel computer-assisted sperm analysis (CASA) system using multitarget-tracking algorithms. *Fertil Steril.* 2010;93:1911–20.
24. Donnelly ET, Lewis SEM, McNally JA, et al. In vitro fertilization and pregnancy rates: The influence of sperm motility and morphology on IVF outcome. *Fertil Steril.* 1998;70:305–14.
25. Davis R. The promise and pitfalls of computer aided sperm analysis. In: Infertility and Reproductive Medicine. Clinics of North America, Diamond MP, DeCherney AH, Overstreet JW (eds.). Philadelphia, PA: WB Saunders, pp. 341–52, 1992.
26. Irvine DS. The computer assisted semen analysis systems: Sperm motility assessment. *Hum Reprod.* 1995;10(Suppl 1):53–9.
27. Krause W. Computer assisted semen analysis systems: Comparison with routine evaluation and prognostic value in male fertility and assisted reproduction. *Hum Reprod.* 1995;10(Suppl 4):60–6.
28. Marshburn PB, Kutteh WH. The role of antisperm antibodies in infertility. *Fertil Steril.* 1994;61:799–811.
29. Golumb J, Vardinon N, Hommonnai ZT, et al. Demonstration of antispermatozoal antibodies in varicocelerelated infertility with an enzyme-linked immunosorbent assay (ELISA). *Fertil Steril.* 1986;45:397–405.
30. Helmerhorst FM, Finken MJ, Erwich JJ. Detection assays for antisperm antibodies: What do they test? *Hum Reprod.* 1999;14: 1669–71.
31. Bronson R. Detection of antisperm antibodies: An argument against therapeutic nihilism. *Hum Reprod.* 1999;14:1671–73.
32. World Health Organization. Manual for Examination of Human Semen and Semen–Cervical Mucus. Cambridge: Cambridge University Press, pp. 1–12, 1987.
33. Noiles EE, Ruffing NA, Kleinhans FW, et al. Critical tonicity determination of sperm using dual fluorescent staining and flow cytometry. In: *Reproduction in Domestic Animals*, (Suppl 1) Boar Semen Preservation II. Proceedings of the Second International Conference on Boar Semen Presentation. Johnson LA, Rath D (eds.). Beltsville, MD: Paul Parey, pp. 359–64, 1991.
34. Noiles EE, Mazur P, Watson PF, et al. Determination of water permeability coefficient for human spermatozoa and its activation energy. *Biol Reprod.* 1993;48:99–109.
35. El-Nour AM, Al Mayman HA, Jaroudi KA, et al. Effects of the hypo-osmotic swelling test on the outcome of intracytoplasmic sperm injection for patients with only nonmotile spermatozoa available for injection: A prospective randomized trial. *Fertil Steril.* 2001;75:480–4.
36. Chan SYW, Fox EJ, Chan MMC. The relationship between the human sperm hypoosmotic swelling test, routine semen analysis, and the human sperm zona free hamster ovum penetration test. *Fertil Steril.* 1985;44:688–92.
37. Jeyendran, RS, Van der Ven HH, Perez-Pelaez M, et al. “Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics.” *Reproduction* 70.1 (1984): 219–228.
38. Joshi N, Kodwany G, Balaiah D, et al. The importance of CASA and sperm function testing in an *in vitro* fertilization program. *Int J Fertil Menopausal Stud.* 1996;41:46–52.
39. Critser JK, Noiles EE. Bioassays of sperm function. *Semin Reprod Endocrinol.* 1993;11:1–16.
40. Yanagimachi R. Mammalian fertilization. In: The Physiology of Reproduction, 2nd ed. Knobil E, Neill JD (eds.). New York, NY: Raven Press, pp. 189–317, 1994.
41. Holden CA, Hyne RV, Sathananthan AH, et al. Assessment of the human sperm acrosome reaction using concanavalin a lectin. *Mol Reprod Dev.* 1990;25:247–57.
42. Liu DY, Clarke GN, Martic M, et al. Frequency of disordered zona pellucida (ZP)-induced acrosome reaction in infertile men with normal semen analysis and normal spermatozoa-ZP binding. *Hum Reprod.* 2001;16:1185–90.
43. Oehninger S, Franken DR, Sayed E, et al. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: A meta-analysis. *Hum Reprod Update.* 2000;6:1160–8.
44. Chan PJ, Corselli JU, Jacobson JD, et al. Spermac stain analysis of human sperm acrosomes. *Fertil Steril.* 1999;72:124–8.
45. Kennedy WP, Kaminski JM, Van der Ven HH, et al. A simple clinical assay to evaluate the acrosin activity of human spermatozoa. *J Androl.* 1989;10:221–31.
46. Mohsenian M, Syner FN, Moghissi KS. A study of sperm acrosin in patients with unexplained infertility. *Fertil Steril.* 1982;37: 223–9.
47. Huszar G, Vigue L, Morshedi M. Sperm creatinine phosphokinase M-isoform ratios and fertilizing potential of men: A blinded study of 84 couples treated with *in vitro* fertilization. *Fertil Steril.* 1992;57:882–8.
48. Yanagimachi R, Yanagimachi H, Rogers BJ. The use of zona-free animal ova as a free system for the assessment of their fertilizing capacity of human spermatozoa. *Biol Reprod.* 1976;15:471–6.
49. Aitken RJ, Thatcher S, Glasier AF, et al. Relative ability of modified versions of the hamster oocyte penetration test, incorporating hyperosmotic medium of the ionophore A23187 to predict IVF outcome. *Hum Reprod.* 1987;2:227–31.
50. Jacobs BR, Caulfield J, Boldt J. Analysis of TEST (TES and tris) yolk buffer effects on human sperm. *Fertil Steril.* 1995;63:1064–70.
51. Mao C, Grimes DA. The sperm penetration assay: Can it discriminate between fertile and infertile men? *Am J Obstet Gynecol.* 1988;159:279–86.
52. O’Shea DL, Odem RR, Cholewa C, et al. Long-term follow-up of couples after hamster egg penetration testing. *Fertil Steril.* 1993;60:1040–5.
53. Oehninger S, Franken DR, Sayed E, et al. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: A meta-analysis. *Hum Reprod Update.* 2000;6:160–8.
54. ESHRE Andrology Special Interest Group. Consensus workshop on advanced diagnostic andrology techniques. *Hum Reprod.* 1996;11:1463–79.
55. Burkman LJ, Coddington CC, Franken DR, et al. The hemizona assay (HZA): Development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril.* 1988;49:688–97.

56. Oehninger S, Acosta AA, Marshedi M, et al. Corrective measures and pregnancy outcome in *in vitro* fertilization in patients with severe sperm morphology abnormalities. *Fertil Steril*. 1989;50:283–7.
57. Liu DY, Baker HW. High frequency of defective sperm–zona pellucida interaction in oligozoospermic infertile men. *Hum Reprod*. 2004;19:228–33.
58. Coddington CC, Oehninger SC, Olive DL, et al. Hemizona index (HZA) demonstrates excellent predictability when evaluating sperm fertilizing capacity in *in vitro* fertilization patients. *J Androl*. 1994;15:250–4.
59. Arslan M, Morshed M, Arslan EO, et al. Predictive value of the hemi zona assay for pregnancy outcome in patients undergoing controlled ovarian hyperstimulation with intrauterine insemination. *Fertil Steril*. 2006;85:1697–707.
60. Mori K, Daitoh T, Irahara M, et al. Significance of d-mannose as a sperm receptor site on the zona pellucida in human fertilization. *Am J Obstet Gynecol*. 1989;161:207–11.
61. Mori K, Daitoh T, Kamada M, et al. Blocking of human fertilization by carbohydrates. *Hum Reprod*. 1993;8:1729–32.
62. Tesarik J, Mendoza C, Carreras R. Expression of d-mannose binding sites on human spermatozoa: Comparison of fertile donors and infertile patients. *Fertil Steril*. 1991;56:113–18.
63. Benoff S, Cooper GW, Hurley I, et al. Human sperm fertilizing potential *in vitro* is correlated with differential expression of a head-specific mannose ligand receptor. *Fertil Steril*. 1993;59:854–62.
64. Silverberg K, Dey T, Witz C et al. D-Mannose binding provides a more objective assessment of male fertility than routine semen analysis: Correlation with *in vitro* fertilization. Presented at the 49th Annual Meeting of the American Fertility Society, October 11–14, 1993, Montreal, Canada.
65. Agarwal A, Said T. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update*. 2003;9:331–45.

SPERM PREPARATION TECHNIQUES AND ADVANCED SPERM SELECTION FOR INTRACYTOPLASMIC SPERM INJECTION

Sinan Ozkavukcu, George Hughes, and Christopher LR Barratt

Overview

Sperm selection *in vivo*

Spermatozoa ejaculated during sexual intercourse undergo a challenging selection process *in vivo* [1]. As shown in humans and many other species, the number of spermatozoa reaching the uterine tubes is tens of thousands of times less than the number ejaculated [2]. The enhanced functional ability of the select cohort of spermatozoa that reach the uterine tubes is attributed to the sequence of events involved in capacitation and hyperactivation [3]. A combination of the challenging anatomical pathway created by the female reproductive tract and sperm competition contributes to eliminating the less functional spermatozoa [1]. Active and passive barriers present in the female reproductive tract, for example, the vaginal pH, cervical mucus, indistinct orifices of the uterine tubes, and the immune responses to the sperm, all aid in selecting a single spermatozoon for fertilization of the oocyte [4–6]. There is preliminary evidence that the accumulation of a higher number of spermatozoa in the ipsilateral uterine tube with the ovulating ovary was attributed not only to the peristaltic contractions in the uterine canal [7, 8] but also to the increased adnexal temperature and hormone concentrations during the late follicular phase [9]. For this reason, the separation of sperm with taxis methods (a form of sperm guidance towards or away from a specified stimulus, e.g., chemotaxis, thermotaxis) has become an increasingly popular way of improving sperm selection *in vitro* [10, 11].

Considering the routine practical applications of today's assisted conception laboratory, many complex mechanisms mentioned earlier can be bypassed to achieve fertilization and help couples dealing with infertility. Although conflicting data exist regarding the fact that the quality of spermatozoa that pass the cervix, uterus, and uterine tubes tend to be more functionally competent than the raw ejaculated sperm, the *in vitro* methods that are applied to isolate the best sperm cohort for *in vitro* fertilization (IVF) are called sperm selection/preparation methods.

Regardless of the insemination method applied in the laboratory, current sperm selection methods can isolate the most favourable cohort of spermatozoa within the heterogeneous population in semen and mathematically increase the chance of selecting the sperm with a high fertilization capacity [12].

Removal of the spermatozoa from the seminal plasma

The process of sperm washing aims to separate spermatozoa from the seminal plasma and thus remove decapacitating and damaging factors for the sperm. Apart from sperm selection, to perform sperm function tests correctly, the seminal plasma and spermatozoa should be separated as soon as liquefaction occurs. Long-term incubation of spermatozoa in seminal plasma increases

their exposure to potentially detrimental factors, mainly caused by non-spermatogenic cells (e.g., immune cells), immature spermatogenic fractions, epithelial cells and cytoplasmic residues, via radical oxygen species (ROS) [13]. ROS products, which are beneficial to some extent for sperm capacitation, can cause damage when they accumulate at pathologic levels [14]. Indeed, a recent publication by Torra-Massana et al. demonstrated adverse clinical outcomes when sperm washing is delayed after collection. The study concluded that the optimum incubation time before sperm washing was 20 minutes, with significantly reduced fertilization rates in both donor and patient oocytes if this period was exceeded [15]. Other publications displayed similar findings in clinical outcomes. Evidence shows that once semen is washed and seminal fluid is separated from the sperm cells, further incubation of spermatozoa in a physiological solution does not cause additional damage [16–21].

Semen, unlike other body fluids, is not in a homogeneous suspension and is made up of contributions from several secretory organs. Ejaculation, a two-stage neuroendocrine reflex, occurs through the successive steps of emission and expulsion. While the seminal vesicle contributes 70% of the volume of semen, it is thought that the prostate and epididymis share the other 30% of the volume equally. Various cellular and biochemical studies have shown that the initial emission into the ejaculatory duct just before expulsion is a mixed secretion of the contents originating from the prostate and the epididymis tail, where the latter is the source of matured spermatozoa [22–26]. Therefore, the first ejaculated fraction is followed by high-volume secretion of the seminal vesicle with very few spermatozoa. During sexual intercourse, following ejaculation, the cervical mucus is washed with the sperm-rich first fraction, and the sperm is instantly carried through the uterus. This physiological process differs significantly from the semen collection procedure. In a clinical setting, the whole semen sample is collected into a container by masturbation, and exposure to seminal vesicle secretions might jeopardise the physiological capacity of the spermatozoa. Studies comparing the utility of split ejaculate fractions have gathered conflicting results, and the technique is not seen as practical in terms of semen collection in clinics [24, 26–28].

Sperm washing

It is recommended to centrifuge the semen to separate seminal plasma from cellular components after dilution with an appropriate medium. Sperm washing is not a sperm selection method as it does not separate live from dead and dying spermatozoa, cellular debris, bacteria, epithelial cells, or immune cells; instead, it collects them all as a packed pellet at the bottom of the centrifuge tube. It is, however, a simple method to remove the seminal plasma containing ROS, prostaglandins, toxicants, and any other contaminants (e.g., gradient medium, fixatives, antibodies,

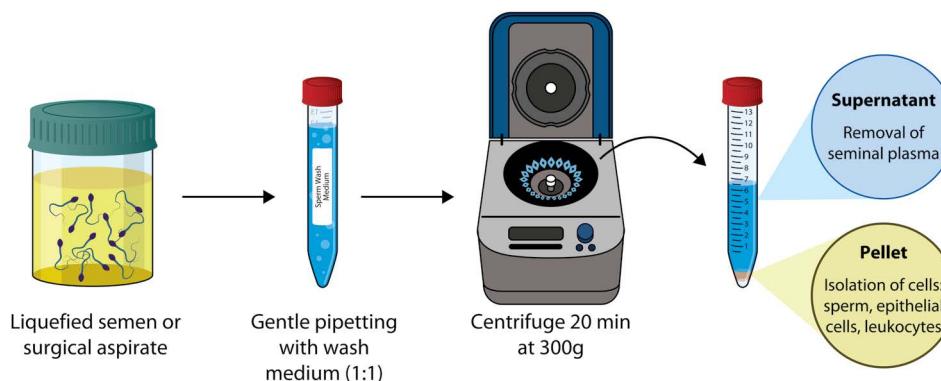


FIGURE 7.1 Basic methodology of sperm washing.

dyes) that are needed to be washed out. Sperm washing is easy to perform before several diagnostic examinations and before and after sperm cryopreservation [29]. A stand-alone sperm washing can be adapted for patients with an extremely low number of ejaculated sperm (e.g., severe oligozoospermia, cryptozoospermia), retrograde ejaculation, or cases with testicular/epididymal aspirations. The basic procedure of sperm washing is depicted in Figure 7.1.

Is centrifugation detrimental to spermatozoa?

Many publications state that the centrifugation method used during sperm preparation compresses the cells with the applied g-force, causing membrane and mitochondrial damage within the cells accumulated in the pellet and thus increasing the production of ROS [30]. It is unclear whether ROS production is caused directly by the sperm cells or other cell types in the semen [31]. It is suggested that the duration of centrifugation rather than the centrifugation speed leads to higher ROS production [32].

It is paradoxical that the centrifugation process, which is used to eliminate the existing ROS in the seminal plasma, is responsible for the production of ROS itself. However, since seminal plasma also contains antioxidant substances, it is known that its removal by centrifugation decreases the elimination of superoxide anion [33]. The current consensus includes choosing a sperm selection method in which the centrifuge step is minimalized or removed.

Sperm selection methods *in vitro*

Conventional sperm selection methods in IVF: Swim-up and density gradient centrifugation *Swim-up*

The swim-up procedure imitates the natural process of spermatozoa migration through the cervical mucus. It is a commonly used technique in IVF laboratories to recover a pure fraction of highly motile sperm with tiny debris, leukocytes, or germ cells. A very rapid direct swim-up can be applied to the ejaculate, where the liquefied semen can be placed into the bottom of a centrifuge tube and overlaid directly with a sperm wash medium. As the sperm swim out of the seminal plasma, motile sperm are collected in the upper-layer culture medium, which leaves the lower

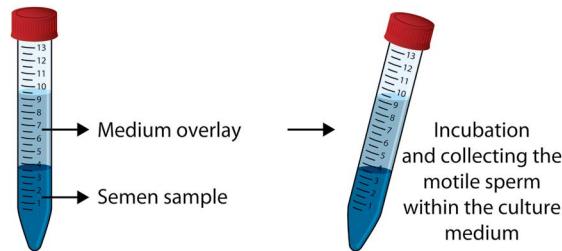


FIGURE 7.2 Rapid isolation of motile sperm using the direct swim-up technique.

layer containing debris, seminal plasma, and immotile sperm (Figure 7.2). The culture media is then pipetted out carefully to gather motile sperm in the final sperm preparation in this direct method [34].

Another method of the swim-up procedure is to wash the semen sample, remove the supernatant after centrifugation, and gently overlay the pellet with a fresh sperm wash medium. The centrifuge tube can be incubated at 37°C at an angle of 45° (to increase the surface area of migration) for 45–60 minutes. Still, the optimum period should preferably be adapted according to the initial sperm concentration and motility. Following incubation, the top portion of the overlay is used for the desired insemination method (Figure 7.3).

Swim-ups are unsuitable for oligo- and asthenozoospermic samples as they may yield a very low number of motile sperm. Highly viscous samples can also respond poorly to the swim-up technique.

Density gradient centrifugation (DGC)

Gradient separation techniques are simple and rapid methods to remove spermatozoa from the seminal plasma and are routinely used for sperm selection for IUI, IVF, and ICSI [35]. Density gradient centrifugation (DGC) and swim-up (SU), which are accepted as the basic sperm preparation methods, have been compared in many studies. Their superiority over each other has been reported in various publications. DGC consists of centrifugation of semen overlaid on the density gradient layer(s) containing silane-coated colloidal silica. Centrifuge forces migrate the cells and accumulate them in different gradient levels by density. Normal sperm have a greater density (1.10 g/mL) than abnormal sperm due to

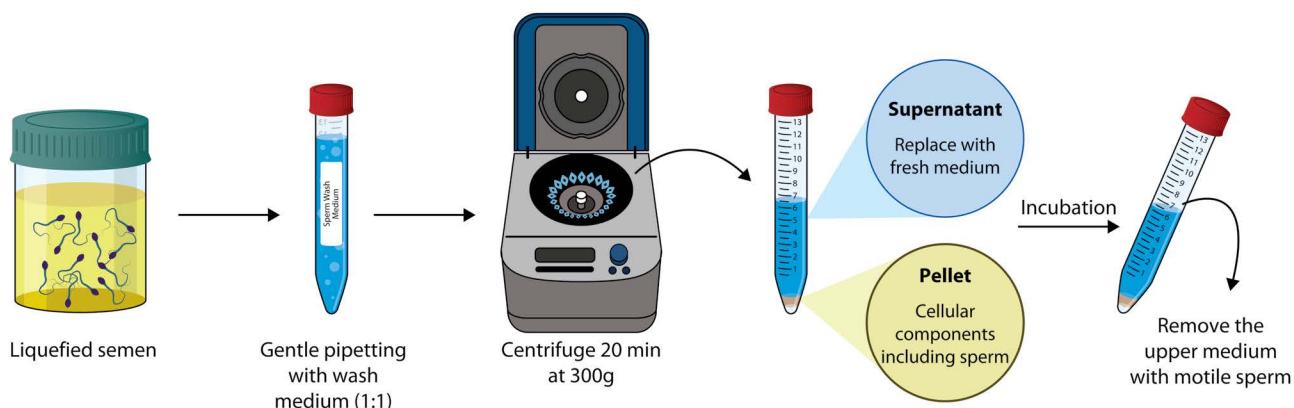


FIGURE 7.3 Swim-up methodology.

their highly condensed DNA. This is known as isopycnic centrifugation, as cells are accumulated at the point where their density is identical to the gradient media [36]. Sperm preparation using DGC usually results in a fraction of highly motile spermatozoa, a cohort free from debris, contaminating leukocytes, non-germ cells, and degenerating germ cells. A simple two-step discontinuous density-gradient preparation method is widely applied, usually following a washing step and with or without a further swim-up step session. Briefly, two different concentrations of gradient media (mostly 45%–90% or 40%–80%) are gently layered as a column to the bottom of a conical centrifuge tube, and the same volume of raw semen is overlaid on the top of them. Solutions can be prepared in desired concentrations, but commercial ready-to-use products are widely available. After centrifugation, a clean sperm suspension, free of seminal plasma, is obtained as a pellet which needs to be washed using a sperm wash medium to remove any trace of the gradient solution (Figure 7.4). In case of a very low number of sperm in the semen, a single layer (continuous) or reduced-volume discontinuous DGC (mini-DGC) can be used to increase the number of recovered sperm in the pellet. It is uncertain if this approach has an additional benefit in semen with abnormal parameters [37, 38].

Comparison of assisted reproductive technique (ART) outcomes after DGC and SU are insufficient due to the limited number of studies with limited sample sizes. A recent

Cochrane meta-analysis included four RCTs to compare clinical pregnancy rates (CPR) after artificial insemination with the semen sample prepared using DGC or SU. There was no significant difference in CPR between the SU and DGC groups [39]. Similarly, a recent study compared cumulative live birth rates (CLBR) after using DGC- or swim-up-prepared sperm and found no significant difference between the groups [40]. Density gradients can be used for normozoospermic samples and those with sub-optimal parameters. They will generally result in high yields of motile sperm, even when samples have an initial low sperm concentration [41].

There is currently insufficient evidence to demonstrate improved results of IVF and ICSI cycles using different sperm preparation methods [40]. However, DGC and SU methods are established as the gold standard for sperm selection/preparation for insemination. To determine the benefits of the new and advanced sperm selection methods mentioned in this chapter, comparative studies should be examined meticulously against these routine methods.

Preparation of surgically aspirated/extracted samples

Epididymal or testicular aspirate fluid can be obtained from the male genital tract by microsurgical epididymal sperm aspiration (MESA), percutaneous epididymal sperm aspiration (PESA), testicular sperm aspiration (TESA), and testicular sperm extraction

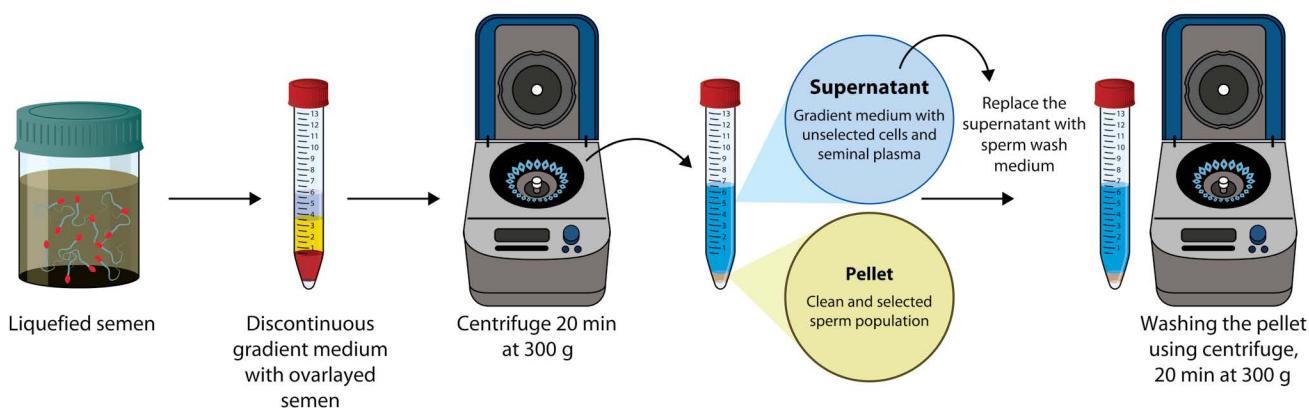


FIGURE 7.4 Steps for discontinuous density gradient centrifugation procedure.

(TESE). If the seminiferous tubules are collected by examining them under high magnification using a stereomicroscope during the TESE operation, it is called a micro-TESE (mTESE) procedure. It is generally acceptable to perform a diagnostic aspiration/extraction before ovarian stimulation and perform freezing to ensure that spermatozoa will be available on the day of oocyte collection for an ICSI treatment cycle. Alternatively, surgical sperm retrieval may be performed on the day of the oocyte retrieval procedure due to poorer freeze–thaw rates of the lower-quality sperm in cases of non-obstructive azoospermia [42].

Epididymal aspirates containing large numbers of motile spermatozoa, with minimal red blood cell and non-germ cell contamination, can be subjected to a DGC as a preparation method for subsequent use. However, a simple wash may also be preferable if a very low number of motile spermatozoa are expected [35].

To free the seminiferous tubule-bound elongated spermatids (“testicular spermatozoa”), enzymatic or mechanical methods are required. The enzymatic method involves incubating the testicular tissue with collagenase for 1.5–2 hours at 37°C and vortexing the suspension every 30 minutes. Centrifugation follows the incubation at 100 g for 10 minutes and a microscopic examination of the pellet [43–45].

The mechanical method involves maceration of the testicular tissue in a culture medium using either glass coverslips or fine needles (attached to disposable tuberculin syringes) that are bent parallel to the base of the culture dish until a fine slurry of dissociated tissue is produced [46]. It is recommended to use the DGC method to obtain clean preparations after the dissection of testicular extraction samples as they contain highly heterogeneous cells and connective tissue components.

Preparation of samples with potential viral load

Using new and improved anti-viral medications has increased life expectancy and quality of life for patients with blood-borne viruses (BBVs). The management of patients with a viral infection/disease in a fertility clinic aims to provide appropriate treatment options that will result in an increased chance of pregnancy whilst reducing the risk of horizontal (person to person) and vertical (mother to the baby) viral transmission.

In patients with a viral infection/disease, it is recommended that in serodiscordant couples (where one partner is BBV-positive and the other partner is uninfected) and when both partners test positive for hepatitis B virus (HBV), hepatitis C virus (HCV) or human immunodeficiency virus (HIV), that the cause of infertility should dictate the specific technique (IUI/IVF/ICSI) used for ART [47–53].

Specific semen preparation procedures have been recommended to reduce or eradicate viral load in treating infected individuals [47, 49, 54, 55]. Patients with BBV who seek fertility treatment must consult an infectious disease specialist before commencing any treatment, as the theoretical risk of vertical transmission remains a possibility.

Currently, no semen preparation techniques can select HBV DNA-free spermatozoa, and current evidence does not recommend HBV DNA testing on seminal fluid or sperm [47, 56–59]. A discontinuous DGC, washing and then swim-up are recommended for semen processing in patients positive for HCV [51, 60–68], especially with a double washing step for patients positive for HIV [69–74]. Regardless of the semen processing technique used, the post-preparation sample that will be used in ART from males who tested positive for HIV should be tested using polymerase chain reaction (PCR). In serodiscordant couples with

the male testing positive for HIV, only an HIV-negative tested sperm sample should be used for the treatments. However, after advanced semen processing, PCR testing for HCV is not necessary. No studies were identified comparing routine semen preparation with advanced semen processing in males testing positive for Human T-lymphotropic virus I/II or Zika virus. For further reading, refer to ESHRE's guideline for medically assisted reproduction in patients with a viral infection or disease [47].

Sperm yield in patients with retrograde ejaculation

Retrograde ejaculation occurs when semen is ejected into the bladder during ejaculation, resulting in aspermia or hypospermia. This can be caused by uncontrolled diabetes mellitus; neurological conditions; side effects of certain drugs; or following prostate, abdominal, pelvic surgery, and radiotherapy. Urine is cytotoxic to spermatozoa due to its high osmolarity and low pH, and as such, in treatment cycles, spermatozoa should be rapidly retrieved from the urine [75–77]. Although alpha-adrenergic agonists, anticholinergic and antihistamine drugs have been described among the medical treatment options in retrograde ejaculation, the preferred method of obtaining spermatozoa is retrieving them from the patient's post-ejaculatory urine. Patients are pre-medicated to make the urine alkaline, using oral sodium bicarbonate or sodium chloride, 1 to 2 hours before attempting to collect an ejaculate. The pre-medication neutralises the highly acidic pH that spermatozoa are exposed to in urine samples, which increases the chance that any spermatozoa will retain their viability and motility characteristics [78]. When the patient is admitted to the andrology laboratory for sample collection, he is given a container for semen production. A second container with 9 mL of sperm washing medium is kept ready at 37°C. In case of ejaculation-with-aspermia, a urine sample is requested into the container containing the sperm washing medium. This sample is immediately distributed to several tubes and centrifuged at 300 g for 10 minutes. After centrifugation, all the supernatant fractions are discarded, and the pellets are collected into a sterile tube with a fresh sperm washing medium. The final suspension is evaluated under the microscope for the presence of viable spermatozoa. For the cells to be used in the ART setting, a double-layered DGC is also recommended [79]. A centrifuge speed of 700 g is suggested by Jarupoonpol et al., yielding a higher number of total motile spermatozoa without an increased risk of DNA fragmentation evaluated by the TUNEL assay [80].

Sperm selection in samples with no motility

Total asthenozoospermia is a rare condition but is still seen in low-quality semen parameters, systemic diseases and after freezing and thawing of the semen. If all sperm are immotile in the ejaculate, the possibility of inappropriate semen collection must first be considered. Using detergent-based lubricants, soap, etc., during masturbation is the most common cause of total asthenozoospermia in clinics. If this is the case, it is advisable to consult the patient and provide clear instructions for semen sample production. Some lubricants in the market are shown to be non-toxic to spermatozoa; thus, their usage in need is reported to be safe during infertility treatments [81].

It is recommended to perform vitality tests if the sperm motility is lower than 40% in repetitive semen analysis, according to the World Health Organization's laboratory manual for the examination and processing of human semen [35]. A motility stimulation test using phosphodiesterase inhibitors (PDEI) on a wet preparation might help diagnose and decide further interventions

during the insemination. In some rare genetic mutations, like in Kartegener's syndrome, structural defects in the ciliary axoneme cause total asthenozoospermia. A definitive diagnosis can be made by extensive history taking, genetic mutation screening, and examination of the ciliary structures under a transmission electron microscope. The only treatment option for these patients is through the use of ICSI, and different methods are suggested to allow the identification of viable sperm. In the presence of PDEI-resistant cases, the use of electrical stimulation [82], hypoosmotic swelling [83], laser-assisted viability assessment [84], and evaluating sperm head birefringence properties [85] are suggested.

Magnetic-activated cell sorting (MACS)

Phosphatidylserine (PS) is a phospholipid found extensively in eukaryotic plasma membranes, and the enzyme flippase typically stabilises PS head regions on the inner layer of the cell membrane. As an early response in biological processes such as apoptosis and necroptosis, the cell surface enzyme scramblase releases the PS and flips its head region to face outside the membrane [86]. The caspase-mediated apoptotic PS exposure is irreversible [87]. Externalized PS (EPS) can be detected by annexin-V immunolabeling, which has a high affinity to PS. The magnetic-activated cell sorting (MACS) technique was designed on the principle of selecting out apoptotic sperm (annexin-V positive cells) (Figure 7.5).

Studies evaluating the efficacy of MACS as a sperm selection method reveal mixed results. In addition to studies comparing MACS versus routine sperm selection techniques, there are studies examining in which order it should be applied. It is reported that when MACS is utilized with DGC, a mean reduction of 70% in EPS-positive sperm is achieved, with an improvement in the viability and motility of the suspension [88]. Other studies have found it more efficient when MACS is performed before DGC [89–91]. Bucar et al. demonstrated that a combination of MACS followed by DGC and swim-up yielded a final sperm preparation

with a low level of DNA fragmentation [92]. Some studies have evaluated the use of MACS in a group of selected patients and have reported favourable clinical outcomes when used in patients with high DNA fragmentation [93–95], teratozoospermia [94, 96, 97], varicocele, and couples with unexplained fertility [98]. A recent randomized controlled trial revealed significantly higher ongoing pregnancy rates using MACS-selected sperm compared to DGC. However, the miscarriage rates were comparable [99]. Sánchez-Martín et al. reported that irrespective of the sperm DNA fragmentation rates in males, MACS significantly decreased the miscarriage rates in ICSI cycles with autologous or donor oocytes [100]. There are studies where no difference is found when sperm are selected with MACS vs routine sperm preparation methods in terms of motility, morphology and DNA fragmentation rates [101], live birth [102], and miscarriage rates [100]. A controlled randomized trial including oocyte donation cycles and unselected males revealed that reproductive outcomes did not improve when MACS was conducted before ICSI [103].

To conclude, there is insufficient evidence to recommend the MACS method to be used alone or in combination with routine procedures in sperm selection. A recent Cochrane database systematic review reported that the beneficial potential of MACS on clinical pregnancy, live birth, and miscarriage rates is uncertain, and the quality of evidence is very low [105]. In a randomized controlled trial, where the obstetric and perinatal outcomes of MACS were evaluated, it was stated that no perinatal adverse side effects were observed [106]. In light of these results, more clinical studies in different indications are needed for sperm selection with MACS, as short-term side effects seem relatively safe.

Microfluidic-based methods for sperm selection

Microfluidic systems have been developed to imitate the sperm movements in the female reproductive tract, the cervical canal, uterine tube lumen, and the complex epithelium of the uterine tubes to isolate a cohort of sperm that have high

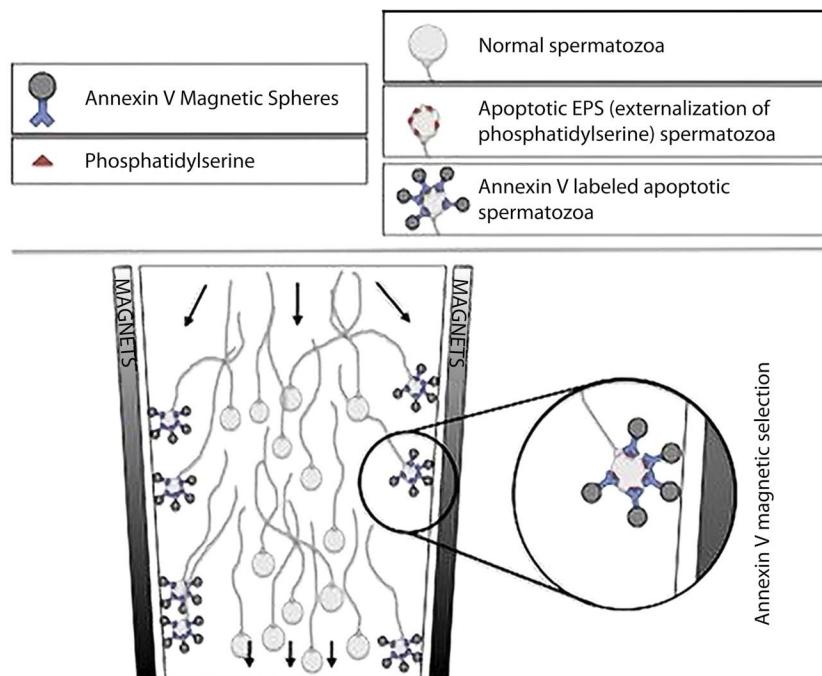


FIGURE 7.5 The principle of sperm selection using magnetic-activated cell sorting. (Modified from [104], with permission.)

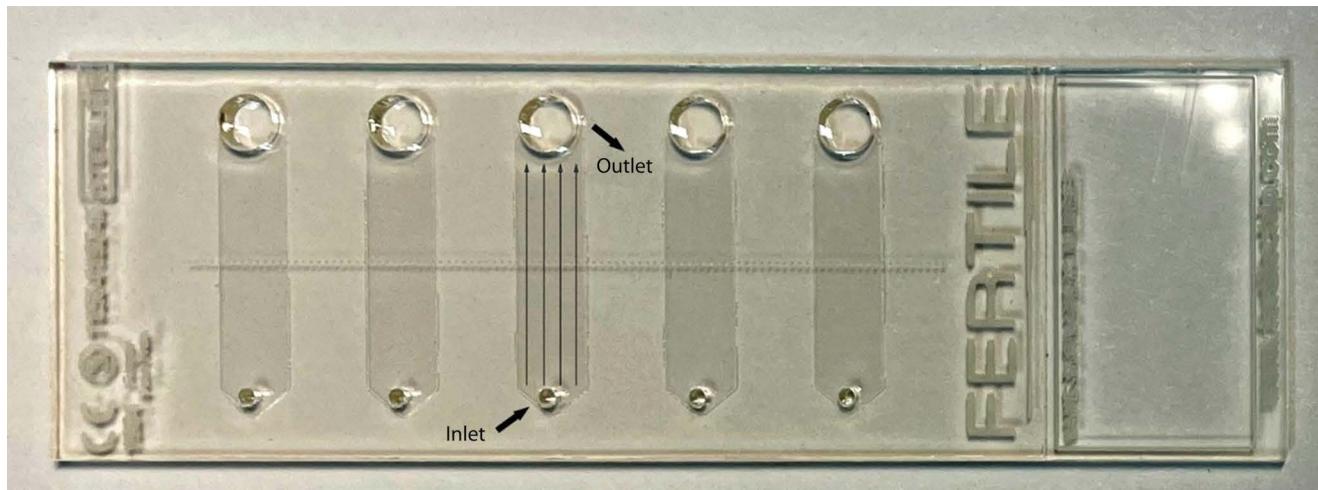


FIGURE 7.6 A commercial product introduced for sperm selection based on the motility of sperm. After the injection of unselected sperm suspension into inlets, by swimming through the microchannels (thin arrows), selected sperm can be collected from the outlet.

fertilization potential. Some of these systems create an actual fluidic environment and support sperm selection over different flow gradients and biophysical forces. However, most devices currently in the market aim to create static microchannel pathways, making selections based on the ability of sperm to pass through labyrinth-like paths depending on their motility (Figure 7.6).

Another strategy used in microfluidic sperm selection is taxis-mediated applications based on the sperm's ability to sense and respond to some external physical stimuli. While the female reproductive tract unites the sperm with the cumulus–oocyte complex (COC) and selects the sperm population with the highest capacity and motility, it has become a fascinating topic for developing novel microfluidic devices [107]. Some mediators, such as progesterone, may increase human sperm capacitation and also have chemotactic properties [108]. Progesterone's ability to activate CatSper channels and to increase Ca^{2+} influx in humans has been shown [107]. Some factors released from the ovulated COC have brought up chemotaxis in developing new microfluidic devices [108–114].

Liquid current forces created in a microfluidic environment also reveal the tendency of the sperm to swim against resistance towards the opposite direction of the current. This phenomenon, called rheotaxis, aims to simulate the indistinctive flow that emerges with ciliary action, muscle contractions, and fluid secretion within the lumen of the uterine tubes. When studies on dynamic microfluidic devices are examined, it can be observed that rheotaxis has been the most commonly studied topic. A recently published model by Jeon et al. used a multidimensional-double spiral microfluidic platform with inertial channels. The fluid flow was run by a syringe pump creating a recirculating flow within the microchannels called Dean flow. Compared to DGC-derived cells, a higher number of sperm were isolated, but the motility parameters were comparable with the control groups [115]. Using similar physical dynamics, Vasilescu et al. published striking data on the successful isolation of testicular-derived sperm with low motility and the elimination of the cancer cells. Separating sperm cells from cancer cells could be beneficial for fertility preservation applications [116].

The third physical property used in microfluidic devices is thermotaxis. The temperature difference of 1°C – 2°C between the two ends of the uterine track may create an attraction for sperm that may lead to the expression of some sensory receptors with the capacity to detect temperature gradients. DeToni et al. localized the expression of TRPV1, known as the heat-sensing receptor, in the male gonads and the sperm cells. They determined that the receptor was effective in sensing temperature gradients, and the sensitivity was increased after sperm capacitation [117]. Despite promising results, studies examining sperm selection by thermotaxis are limited, and further data to support the use of this mechanism is required.

Most clinical studies on microfluidic sperm selection are based on the swimming capacity of the sperm throughout microchannels filled with a static fluid. One study published by Gode et al., where IUI cases were inseminated with sperm prepared using a microfluidic device or DGC, highlighted improved pregnancy rates [118]. A study evaluating the ICSI cycle results stated that there was no significant difference in any clinical outcomes among the included couples; however, when a subgroup analysis was performed, more favourable results were evident in cases of advanced maternal age and severe oligozoospermia [119]. Yildiz et al. stated that when couples' first IVF trials were considered, there was no significant difference between the reproductive outcomes when the microfluidic technique was used. Still, a higher pregnancy rate was obtained in the couples who underwent their second IVF cycles [120]. Similarly, it was reported in two different RCTs that there was no significant difference with the control group in terms of selected sperm parameters or the clinical outcomes in couples with unexplained infertility or unselected males when microfluidic sperm selection is performed [121, 122]. In another study, the results on sibling oocytes were evaluated, with no significant difference between the experimental groups [123].

Suffice it to say that there is insufficient data regarding the superiority of microfluidic sperm selection methods in ART cycles. More randomized controlled trials on the use and clinical outcomes of rheo-, thermo-, and chemo-attractants are required.

High-resolution selection of sperm for ICSI (IMSI/MSOME)

Sperm morphology is associated with chromatin maturation defects, DNA fragmentation, aneuploidies, fertilization and embryo development rates, and obstetric outcomes [124]. The association between the long-term well-being status of the offspring with paternal sperm quality has also been shown [125].

Although many methods discussed in this chapter allow the selection of a representative sperm population from raw semen, during the ICSI procedure, the final selection of sperm to be injected into the oocyte is made by the embryologists. In contrast to routine ICSI, where sperm for injection is selected under low-power magnification (200 \times –400 \times), Intracytoplasmic Morphologically Selected Sperm Injection (IMSI) combines ICSI and Motile Sperm Organelle Morphology Examination (MSOME) with high-power ($>6000\times$) optical and digital methods to aid selection of the best available spermatozoa [126]. MSOME detects subtle organellar malformations in sperm by examining six subcellular organelles: acrosome, post-acrosomal lamina, neck, mitochondria, tail, and nucleus of the spermatozoa [126–128]. With this technology, many studies have focused on the clear visualization of vacuoles in the sperm head (Figure 7.7) and instant deselection. Vacuoles in the sperm head may originate from the nucleus, acrosome, or post-acrososomal region, and the small ones cannot be detected under conventional magnifications. Publications suggesting MSOME as a diagnostic tool in identifying sperm head vacuoles revealed various predisposing factors such as advanced male age [129], sperm DNA fragmentation [129], abnormal chromatin packing [130–133], and diminished outcomes like poor fertilization [129], suboptimal embryo development [134], low pregnancy rates [129, 135–137], implantation failure [129, 136, 137], and low live birth rates [136, 137]. Severe male infertility is the primary indication suggested for the selected group of patients [138–144], while no benefit was revealed for the cases of repeated implantation failure [145–148]. Data from randomized clinical trials, evaluated in a recently published meta-analysis, show that IMSI does not provide superiority in live birth or miscarriage rates over conventional sperm selection methods [147]. The data obtained from studies comparing the use of IMSI and conventional ICSI in a Cochrane systematic

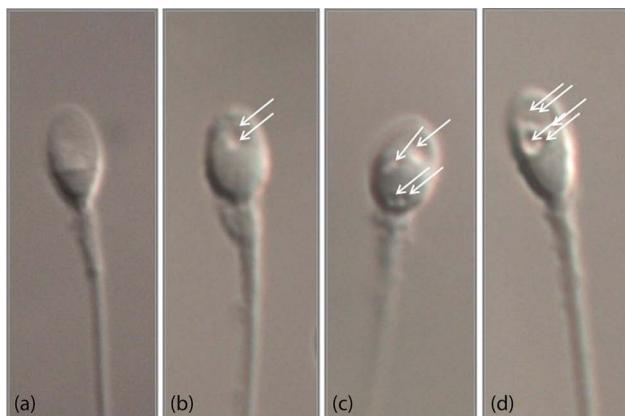


FIGURE 7.7 By using optical and digital high magnifications, sperm morphology (a) can be evaluated for selection during ICSI according to acrosomal (b), nuclear (c), or mixed (d) vacuoles in the sperm head. (From [151], with permission.)

review reported a significant improvement in the clinical pregnancy rates in the presence of very-low-quality evidence but with no apparent significant difference in the miscarriage rates [149]. It is stated that the potential disadvantages of the technique are the increased average duration of the procedures and the cost of essential microscopic equipment [150].

In light of current data, and since it has been shown that the IMSI technique does not have any side effects on neonatal outcomes [152], it is possible that IMSI may be considered as a sperm selection method in cases of severe male factor infertility. However, randomized controlled studies are required to confirm this potential indication.

Sperm selection based on hyaluronic acid binding capacity (P-ICSI)

The binding capacity of mature sperm to hyaluronic acid (HA, hyaluronan) was introduced as a commercial diagnostic kit in the early 2000s as the Hyaluronic acid Binding Assay (HBA). According to this principle, spermatozoa with higher capacity to bind to the zona pellucida, higher fertilization ability, advanced chromatin maturation, lower DNA fragmentation, lower aneuploidy rate, and superior morphology express HA binding sites which attach to HA-coated surfaces from their head region. Thus, with the help of HBA, in addition to the basic semen parameters of the patients, a new diagnostic test is proposed to improve the diagnosis of male factor infertility, particularly in the determination of ICSI indication [153–155].

Although the use of HBA as a screening test for the prediction of ICSI indication is not supported by subsequent studies [156], it has been recommended as a sperm selection method during the ICSI procedure. The method is based on the principle of building solid-state HA-coated areas on the surface of a classical ICSI dish. The sperm with higher potential adhere to these surfaces whilst passing through these areas. The ICSI practitioner collects these sperm prospectively with a microinjection needle and injects them into the oocyte (Figure 7.8).

Physiological selection (or namely “picking”) of spermatozoa for ICSI (P-ICSI) has found a wide area of interest and has been the subject of many clinical studies. P-ICSI-selected sperm have been compared with conventional ICSI groups in couples with recurrent pregnancy loss [157, 158], high DNA fragmentation index [95, 99] and high teratozoospermia [159, 160], and conflicting results have been reported. The largest multicentral randomized clinical trial included 2772 couples in 16 different centres in the UK, was called the HABSelect study. The couples were randomly allocated into conventional ICSI and P-ICSI groups, where the primary outcome was full-term live birth. Miller et al. reported that sperm selection using HA binding provided no difference between the groups regarding live birth rates. As a secondary outcome in the study, miscarriage rates were significantly decreased in the P-ICSI group [161]. A predictive model based on the same database and re-analysis of the frozen samples has been published recently, stating that the use of HA-based selection during ICSI may be beneficial in the treatment of couples with older maternal age [162]. A sibling oocyte study which included 45 IVF cycles of patients with previous fertilization failure, poor embryonic development, implantation failure, or miscarriage, reported significantly improved fertilization and embryo utilization rates when oocytes were fertilized using P-ICSI dishes [163].

The results of studies examining the benefits of HA-based sperm selection during ICSI are highly contradictory. A systematic review found no statistically significant difference between

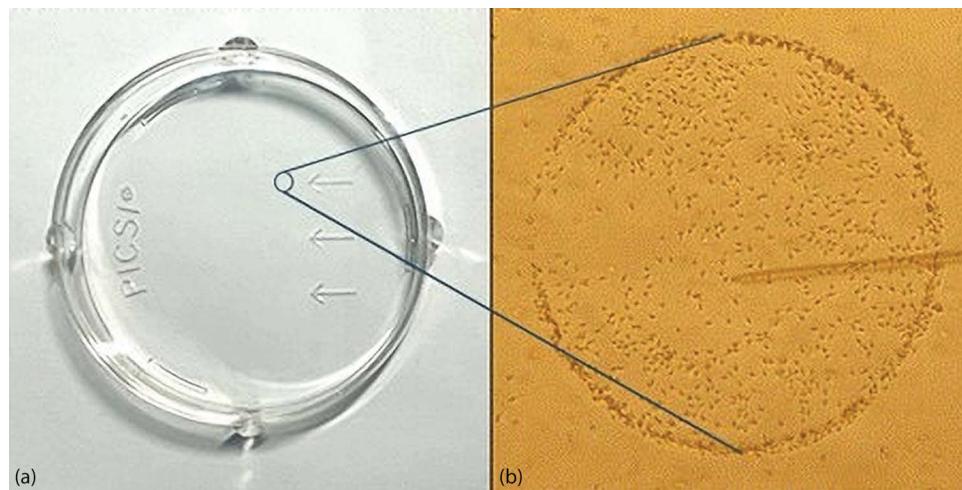


FIGURE 7.8 P-ICSI dish with solid HA-coated areas pointed by arrows (a) allows picking of bounded spermatozoa by the microinjection pipette during ICSI (b). (From [164], with permission.)

P-ICSI and ICSI groups regarding any analysed outcomes [165]. Finally, a meta-analysis reported improved embryo quality and implantation rate, whereas if solely prospective studies were considered, the only improved parameter remains as the embryo quality. Thus, they concluded that the evidence does not support the routine use of hyaluronic acid binding assays in all ICSI cycles, and further studies are required to establish which patients may benefit from this technique [166].

Other sperm selection methods

Sperm selection based on the electrostatic charge

The sperm membrane undergoes many chemical changes during sperm maturation, which are fundamental in maintaining the fertilization potential of the sperm. The maintenance of this physiological function is provided by the membrane-coating glycocalyx containing sialic acid so that the sperm outer membrane carries a negative charge of -16 to -20 mV, which decreases with the process of capacitation. In mammalian species, CD52 is a bipolar glycopeptide of epididymal origin that forms the main component of the sperm glycocalyx and is responsible for the net negative charge when transferred to the sperm membrane. It was reported that sperm with a negative charge on their membrane is significantly higher in fertile men when compared to ones who are sub-fertile [167]. A positively charged tube isolates sperm with a net negative charge in the zeta potential method. In contrast, electrophoresis attracts sperm with a negative charge to a positive electrode when suspended in an electrophoretic buffer. In this suspension, sperm move according to their net charge on the membrane and can pass through separating barriers with 5- μ m pore size so that larger cells, such as immature germ cells and leukocytes, are eliminated in the final preparation [168]. There is a need to determine its effectiveness with detailed comparative clinical studies.

Birefringence

Birefringence is a polarized microscopy method based on the principle that light is refracted when passing through objects with an anisotropic structure; the refracted light delays and creates artificial brightness on the object. Studies show its benefit in selecting spermatozoa in patients with severe oligoasthenozoospermia and

immotile sperm by examining the sperm head with a polarization microscope during ICSI [169]. It has also been reported that it can be used to select testicular sperm, sperm that have undergone acrosome reaction [170], and sperm with total asthenozoospermia [85], but more studies are needed on the technique.

Raman spectroscopy

Although the sperm selection method using Raman spectroscopy is still experimental, it has been the subject of intense research in recent years. By applying lasers and collecting the reflected Raman light spectrum, it is possible to distinguish the composition, crystal symmetry, crystal quality or the amount of supplemented ingredients in a given sample. This data can be used as a non-invasive detection of the sperm head to evaluate nuclear DNA status, identify chromatin damage, and construct maps showing the area where the fragmented DNA is found [171].

Summary

In assisted reproduction techniques, whether with ICSI or cIVF insemination, *in vitro* sperm selection methods are used to increase the chance that a functional spermatozoon is involved in fertilization. Several publications have presented data on which method is more advantageous for this selection; nevertheless, density gradient centrifugation, and swim-up remain as the routine and gold-standard sperm selection methods due to their ease of application and cost. One should be selective in introducing new techniques into the laboratory until basic and clinical studies have demonstrated the method's definitive superiority. Because of the potential commercial value of new techniques, the methodology and results of published studies need to be carefully evaluated. Furthermore, the usefulness of these methods in patients with different diagnoses should be investigated, and specific indications should be determined.

References

1. Sakkas D, et al. Sperm selection in natural conception: What can we learn from mother nature to improve assisted reproduction outcomes? *Hum Reprod Update*. 2015;21(6):711–26.

2. Williams M, et al. Recovery of artificially inseminated spermatozoa from the fallopian tubes of a woman undergoing total abdominal hysterectomy. *Hum Reprod.* 1992;7(4):506–9.
3. Suarez SS, Ho HC. Hyperactivated motility in sperm. *Reprod Domest Anim.* 2003;38(2):119–24.
4. Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. *Hum Reprod Update.* 2006;12(1):23–37.
5. Holt WV, Fazeli A. Do sperm possess a molecular passport? Mechanistic insights into sperm selection in the female reproductive tract. *Mol Hum Reprod.* 2015;21(6):491–501.
6. Holt WV, Fazeli A. The oviduct as a complex mediator of mammalian sperm function and selection. *Mol Reprod Dev.* 2010;77(11):934–43.
7. Kunz G, et al. The dynamics of rapid sperm transport through the female genital tract: Evidence from vaginal sonography of uterine peristalsis and hysterosalpingosintigraphy. *Hum Reprod.* 1996;11(3):627–32.
8. Kunz G, et al. Oxytocin—a stimulator of directed sperm transport in humans. *Reprod Biomed Online.* 2007;14(1):32–39.
9. Zervomanolakis I, et al. Uterine mechanisms of ipsilateral directed spermatozoa transport: Evidence for a contribution of the utero-ovarian countercurrent system. *Eur J Obstet Gynecol Reprod Biol.* 2009;144(Suppl 1):S45–9.
10. Eisenbach M. Mammalian sperm chemotaxis and its association with capacitation. *Dev Genet.* 1999;25(2):87–94.
11. Suarez SS, Wu M. Microfluidic devices for the study of sperm migration. *Mol Hum Reprod.* 2017;23(4):227–34.
12. Shen S, et al. Statistical analysis of factors affecting fertilization rates and clinical outcome associated with intracytoplasmic sperm injection. *Fertil Steril.* 2003;79(2):355–60.
13. Aitken RJ. Impact of oxidative stress on male and female germ cells: Implications for fertility. *Reproduction.* 2020;159(4):R189–201.
14. Aitken RJ, Jones KT, Robertson SA. Reactive oxygen species and sperm function—in sickness and in health. *J Androl.* 2012;33(6):1096–106.
15. Torra-Massana M, et al. How long can the sperm wait? Effect of incubation time on ICSI outcomes. *Mol Reprod Dev.* 2022;89(3):133–45.
16. Ahmed I, et al. Influence of extended incubation time on human sperm chromatin condensation, sperm DNA strand breaks and their effect on fertilisation rate. *Andrologia.* 2018;50(4):e12960.
17. Peer S, et al. Is fine morphology of the human sperm nuclei affected by in vitro incubation at 37°C? *Fertil Steril.* 2007;88(6):1589–94.
18. Calamera JC, et al. Effects of long-term in vitro incubation of human spermatozoa: Functional parameters and catalase effect. *Andrologia.* 2001;33(2):79–86.
19. Matsuurra R, Takeuchi T, Yoshida A. Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. *Asian J Androl.* 2010;12(5):753–9.
20. Mansour RT, et al. The impact of spermatozoa preincubation time and spontaneous acrosome reaction in intracytoplasmic sperm injection: A controlled randomized study. *Fertil Steril.* 2008;90(3):584–91.
21. Zhang X-D, et al. The effects of different sperm preparation methods and incubation time on the sperm DNA fragmentation. *Hum Fertil.* 2011;14(3):187–91.
22. Farris EJ, Murphy DP. The characteristics of the two parts of the partitioned ejaculate and the advantages of its use for intrauterine insemination. *Fertil Steril.* 1960;11(5):465–9.
23. MacLeod J, Hotchkiss RS. The distribution of spermatozoa and of certain chemical constituents in the human ejaculate. *J Urol.* 1942;48(2):225–9.
24. Marmar JL, Praiss DE, Debenedictis TJ. Statistical comparison of the parameters of semen analysis of whole semen versus the fractions of the Split ejaculate. *Fertil Steril.* 1978;30(4):439–43.
25. Skandhan K, et al. Level of copper in human split ejaculate. *Urologia.* 2017;84(1):51–4.
26. Williams CA, Newton JR. A comparison of the properties of the two portion split ejaculate in normal and in oligospermic subjects. *Acta Eur Fertil.* 1978;9(3):145–9.
27. Bjorndahl L, Kvist U. Sequence of ejaculation affects the spermatozoon as a carrier and its message. *Reprod Biomed Online.* 2003;7(4):440–8.
28. Lindholmer C. Survival of human spermatozoa in different fractions of split ejaculate. *Fertil Steril.* 1973;24(7):521–6.
29. Sieme H, Oldenhof H. Sperm cleanup and centrifugation processing for cryopreservation. *Methods Mol Biol.* 2015;1257:343–52.
30. Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl.* 1988;9(6):367–76.
31. Rappa KL, et al. Sperm processing for advanced reproductive technologies: Where are we today? *Biotechnol Adv.* 2016;34(5):578–87.
32. Shekarriz M, et al. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. *Eur Urol.* 1995;28:31–5.
33. Ghaleo LR, et al. Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: A flow cytometric study. *Arch Gynecol Obstet.* 2014;289(1):173–80.
34. Mortimer D. Sperm preparation methods. *J Androl.* 2000;21(3):357–66.
35. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen, 6th edition. Geneva: World Health Organization, 2021.
36. Allamaneni SS, et al. Comparative study on density gradients and swim-up preparation techniques utilizing neat and cryopreserved spermatozoa. *Asian J Androl.* 2005;7(1):86–92.
37. Ord T, et al. Mini-percoll: A new method of semen preparation for IVF in severe male factor infertility. *Hum Reprod.* 1990;5(8):987–9.
38. Ng FL, Liu DY, Baker HW. Comparison of percoll, mini-percoll and swim-up methods for sperm preparation from abnormal semen samples. *Hum Reprod.* 1992;7(2):261–6.
39. Boomsma CM, Cohlen BJ, Farquhar C. Semen preparation techniques for intrauterine insemination. *Cochrane Database Syst Rev.* 2019(10):1–46.
40. Rao M, et al. Cumulative live birth rates after IVF/ICSI cycles with sperm prepared by density gradient centrifugation vs. swim-up: A retrospective study using A propensity score-matching analysis. *Reprod Biol Endocrinol.* 2022;20(1):60.
41. Jeyendran RS, et al. Selecting the most competent sperm for assisted reproductive technologies. *Fertil Steril.* 2019;111(5):851–63.
42. Specialised Commissioning Team. Clinical Commissioning Policy: Surgical sperm retrieval for male infertility, NHS England, England, 2016.
43. Aydos K, et al. *Enzymatic digestion plus mechanical searching improves testicular sperm retrieval in non-obstructive azoospermia cases.* *Eur J Obstet Gynecol Reprod Biol.* 2005;120(1):80–6.
44. Verheyen G, Popovic-Todorovic B, Tournaye H. Processing and selection of surgically-retrieved sperm for ICSI: A review. *Basic Clin Androl.* 2017;27:6.
45. Wöber M, et al. A new method to process testicular sperm: Combining enzymatic digestion, accumulation of spermatozoa, and stimulation of motility. *Arch Gynecol Obstet.* 2015;291(3):689–94.
46. Verheyen G, Popovic-Todorovic B, Tournaye H. Processing and selection of surgically-retrieved sperm for ICSI: A review. *Basic Clin Androl.* 2017;27(1):6.
47. Mocanu E, et al. *ESHRE guideline: Medically assisted reproduction in patients with a viral infection/disease.* *Hum Reprod Open.* 2021;2021(4):hoab037.
48. Nie R, et al. Assisted conception does not increase the risk for mother-to-child transmission of hepatitis B virus, compared with natural conception: A prospective cohort study. *Fertil Steril.* 2019;111(2):348–56.

49. Garrido N, et al. Report of the results of a 2 year programme of sperm wash and ICSI treatment for human immunodeficiency virus and hepatitis C virus serodiscordant couples. *Hum Reprod.* 2004;19(11):2581–6.
50. Nesrine F, Saleh H. Hepatitis C virus (HCV) status in newborns born to HCV positive women performing intracytoplasmic sperm injection. *Afr Health Sci.* 2012;12(1):58–62.
51. Savasi V, et al. Should HCV discordant couples with a seropositive male partner be treated with assisted reproduction techniques (ART)? *Eur J Obstet Gynecol Reprod Biol.* 2013;167(2):181–4.
52. Vitorino RL, et al. Systematic review of the effectiveness and safety of assisted reproduction techniques in couples serodiscordant for human immunodeficiency virus where the man is positive. *Fertil Steril.* 2011;95(5):1684–90.
53. Barnes A, et al. Efficacy and safety of intrauterine insemination and assisted reproductive technology in populations serodiscordant for human immunodeficiency virus: A systematic review and meta-analysis. *Fertil Steril.* 2014;102(2):424–34.
54. Garrido N, et al. First report of the absence of viral load in testicular sperm samples obtained from men with hepatitis C and HIV after washing and their subsequent use. *Fertil Steril.* 2009;92(3):1012–15.
55. Inoue O, et al. Clinical efficacy of a combination of percoll continuous density gradient and swim-up techniques for semen processing in HIV-1 serodiscordant couples. *Asian J Androl.* 2017;19(2):208–13.
56. Ayoola EA, Ladipo OA, Odelola HA. Antibody to hepatitis B core antigen, e-antigen and its antibody in menstrual blood and semen. *Int J Gynecol Obstet.* 1981;19(3):221–3.
57. Hadchouel M, et al. Presence of HBV DNA in spermatozoa: A possible vertical transmission of HBV via the germ line. *J Med Virol.* 1985;16(1):61–6.
58. Qian WP, et al. Rapid quantification of semen hepatitis B virus DNA by real-time polymerase chain reaction. *World J Gastroenterol.* 2005;11(34):5385–9.
59. Fei QJ, et al. Can hepatitis B virus DNA in semen be predicted by serum levels of hepatitis B virus DNA, HBeAg, and HBsAg in chronically infected men from infertile couples? *Andrology.* 2015;3(3):506–11.
60. Bourlet T, et al. Detection and characterization of hepatitis C virus RNA in seminal plasma and spermatozoon fractions of semen from patients attempting medically assisted conception. *J Clin Microbiol.* 2002;40(9):3252–5.
61. Cassuto NG, et al. A modified RT-PCR technique to screen for viral RNA in the semen of hepatitis C virus-positive men. *Hum Reprod.* 2002;17(12):3153–6.
62. Meseguer M, et al. Comparison of polymerase chain reaction-dependent methods for determining the presence of human immunodeficiency virus and hepatitis C virus in washed sperm. *Fertil Steril.* 2002;78(6):1199–202.
63. Canto CL, et al. Detection of HIV and HCV RNA in semen from Brazilian coinfected men using multiplex PCR before and after semen washing. *Rev Inst Med Trop Sao Paulo.* 2006;48(4):201–6.
64. Garrido N, et al. The effectiveness of modified sperm washes in severely oligoasthenozoospermic men infected with human immunodeficiency and hepatitis C viruses. *Fertil Steril.* 2006;86(5):1544–6.
65. Savasi V, et al. Hepatitis C virus RNA detection in different semen fractions of HCV/HIV-1 co-infected men by nested PCR. *Eur J Obstet Gynecol Reprod Biol.* 2010;151(1):52–5.
66. Leruez-Ville M, et al. Intracytoplasmic sperm injection with micro-surgically retrieved spermatozoa in azoospermic men infected with human immunodeficiency virus 1 or hepatitis C virus: The EP43 AZONECO ANRS study. *Fertil Steril.* 2013;99(3):713–7.
67. Molina I, et al. Assisted reproductive technology and obstetric outcome in couples when the male partner has a chronic viral disease. *Int J Fertil Steril.* 2014;7(4):291–300.
68. Bourlet T, et al. Prospective evaluation of the threat related to the use of seminal fractions from hepatitis C virus-infected men in assisted reproductive techniques. *Hum Reprod.* 2008;24(3):530–5.
69. Dussaix E, et al. Spermatozoa as potential carriers of HIV. *Res Virol.* 1993;144:487–95.
70. Baccetti B, et al. HIV-particles in spermatozoa of patients with AIDS and their transfer into the oocyte. *J Cell Biol.* 1994;127(4):903–14.
71. Quayle AJ, et al. T lymphocytes and macrophages, but not motile spermatozoa, are a significant source of human immunodeficiency virus in semen. *J Infect Dis.* 1997;176(4):960–8.
72. Deleage C, et al. Human immunodeficiency virus infects human seminal vesicles in vitro and in vivo. *Am J Pathol.* 2011;179(5):2397–408.
73. Miller RL, et al. HIV diversity and genetic compartmentalization in blood and testes during suppressive antiretroviral therapy. *J Virol.* 2019;93(17):e00755–19.
74. Young CD, et al. Sperm can act as vectors for HIV-1 transmission into vaginal and cervical epithelial cells. *Am J Reprod Immunol.* 2019;82(1):e13129.
75. Makler A, et al., Factors affecting sperm motility. VII. Sperm viability as affected by change of pH and osmolarity of semen and urine specimens. *Fertil Steril.* 1981;36(4):507–11.
76. Chen D, Scobey MJ, Jeyendran RS. Effects of urine on the functional quality of human spermatozoa. *Fertil Steril.* 1995;64(6):1216–7.
77. Aust TR, et al. Development and in vitro testing of a new method of urine preparation for retrograde ejaculation; The Liverpool solution. *Fertil Steril.* 2008;89(4):885–91.
78. Mahadevan M, Leeton JF, Trounson AO. Noninvasive method of semen collection for successful artificial insemination in a case of retrograde ejaculation. *Fertil Steril.* 1981;36(2):243–7.
79. Gupta S, et al. A comprehensive guide to sperm recovery in infertile men with retrograde ejaculation. *World J Mens Health.* 2022;40(2):208–16.
80. Jarupoonpol S, et al. Sperm motility and DNA integrity affected by different g-forces in the preparation of sperm in urine specimens. *Andrologia.* 2014;46(4):391–8.
81. Mackenzie SC, Gellatly SA. Vaginal lubricants in the couple trying-to-conceive: Assessing healthcare professional recommendations and effect on in vitro sperm function. *PLoS One.* 2019;14(5):e0209950.
82. Shin TE, et al. Motility enhancement of human spermatozoa using electrical stimulation in the nano-ampere range with enzymatic biofuel cells. *PLoS One.* 2020;15(2):e0228097.
83. Ortega C, et al. Absolute asthenozoospermia and ICSI: What are the options? *Hum Reprod Update.* 2011;17(5):684–92.
84. Ozkavukcu S, et al. Live birth after laser assisted viability assessment (LAVA) to detect pentoxifylline resistant ejaculated immobile spermatozoa during ICSI in a couple with male Kartagener's syndrome. *Reprod Biol Endocrinol: RB&E.* 2018;16(1):10.
85. Magli MC, et al., Birefringence properties of human immobile spermatozoa and ICSI outcome. *Reprod Biomed Online.* 2022;46(3):597–606.
86. Kagan VE, et al. Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. *FEBS Lett.* 2000;477(1-2):1–7.
87. Segawa K, Suzuki J, Nagata S. Flippases and scramblases in the plasma membrane. *Cell Cycle.* 2014;13(19):2990–91.
88. de Vantéry Arrighi C, et al. Removal of spermatozoa with externalized phosphatidylserine from sperm preparation in human assisted medical procreation: Effects on viability, motility and mitochondrial membrane potential. *Reprod Biol Endocrinol.* 2009;7:1.
89. Chi HJ, et al. Efficient isolation of sperm with high DNA integrity and stable chromatin packaging by a combination of density-gradient centrifugation and magnetic-activated cell sorting. *Clin Exp Reprod Med.* 2016;43(4):199–206.

90. Tavalaee M, et al. Density gradient centrifugation before or after magnetic-activated cell sorting: Which technique is more useful for clinical sperm selection? *J Assist Reprod Genet.* 2012;29(1):31–8.
91. Berteli TS, et al. Magnetic-activated cell sorting before density gradient centrifugation improves recovery of high-quality spermatozoa. *Andrology.* 2017;5(4):776–82.
92. Bucar S, et al. DNA fragmentation in human sperm after magnetic-activated cell sorting. *J Assist Reprod Genet.* 2015;32(1):147–54.
93. Pacheco A, et al. Magnetic-activated cell sorting (MACS): A useful sperm-selection technique in cases of high levels of sperm DNA fragmentation. *J Clin Med.* 2020;9(12):3976.
94. Stimpfle M, et al., Magnetic-activated cell sorting of non-apoptotic spermatozoa improves the quality of embryos according to female age: A prospective sibling oocyte study. *J Assist Reprod Genet.* 2018;35(9):1665–74.
95. Hasanen E, et al. PICSI vs. MACS for abnormal sperm DNA fragmentation ICSI cases: A prospective randomized trial. *J Assist Reprod Genet.* 2020;37(10):2605–13.
96. Dirican EK, et al. Clinical outcome of magnetic activated cell sorting of non-apoptotic spermatozoa before density gradient centrifugation for assisted reproduction. *J Assist Reprod Genet.* 2008;25(8):375–81.
97. Degheidy T, et al. Magnetic activated cell sorting: An effective method for reduction of sperm DNA fragmentation in varicocele men prior to assisted reproductive techniques. *Andrologia.* 2015;47(8):892–6.
98. Lee TH, et al. Magnetic-activated cell sorting for sperm preparation reduces spermatozoa with apoptotic markers and improves the acrosome reaction in couples with unexplained infertility. *Hum Reprod.* 2010;25(4):839–46.
99. Hozyen M, et al. Reproductive outcomes of different sperm selection techniques for ICSI patients with abnormal sperm DNA fragmentation: A randomized controlled trial. *Reprod Sci.* 2022;29(1):220–8.
100. Sánchez-Martín P, et al. Magnetic cell sorting of semen containing spermatozoa with high DNA fragmentation in ICSI cycles decreases miscarriage rate. *Reprod Biomed Online.* 2017;34(5):506–12.
101. Nadalini M, et al. Annexin v magnetic-activated cell sorting versus swim-up for the selection of human sperm in ART: Is the new approach better than the traditional one? *J Assist Reprod Genet.* 2014;31(8):1045–51.
102. Gil Juliá M, et al. Cumulative live birth rates in donor oocyte ICSI cycles are not improved by magnetic-activated cell sorting sperm selection. *Reprod Biomed Online.* 2022;44(4):677–84.
103. Romany L, et al. Removal of annexin V-positive sperm cells for intracytoplasmic sperm injection in ovum donation cycles does not improve reproductive outcome: A controlled and randomized trial in unselected males. *Fertil Steril.* 2014;102(6):1567–75.e1.
104. Valcance DG, et al. Selection of nonapoptotic sperm by magnetic-activated cell sorting in Senegalese sole (*Solea senegalensis*). *Theriogenology.* 2016;86(5):1195–202.
105. Lepine S, et al. Advanced sperm selection techniques for assisted reproduction. *Cochrane Database Syst Rev.* 2019;7(7):Cd010461.
106. Romany L, et al. Obstetric and perinatal outcome of babies born from sperm selected by MACS from a randomized controlled trial. *J Assist Reprod Genet.* 2017;34(2):201–7.
107. Kolle S. Sperm-oviduct interactions: Key factors for sperm survival and maintenance of sperm fertilizing capacity. *Andrology.* 2022;10(5):837–43.
108. Devigili A, Cattelan S, Gasparini C. Sperm accumulation induced by the female reproductive fluid: Putative evidence of chemoattraction using a new tool. *Cells.* 2021;10(9):2472.
109. Tamburrino L, et al. Progesterone, spermatozoa and reproduction: An updated review. *Mol Cell Endocrinol.* 2020;516:110952.
110. Dominguez EM, et al. Chemotactic selection of frozen-thawed stallion sperm improves sperm quality and heterologous binding to oocytes. *Anim Reprod Sci.* 2020;221:106582.
111. Li K, et al. Novel distance-progesterone-combined selection approach improves human sperm quality. *J Transl Med.* 2018;16(1):203.
112. Li J, et al. Separation of motile sperm for in vitro fertilization from frozen-thawed bull semen using progesterone induction on a microchip. *Anim Reprod Sci.* 2016;172:52–9.
113. Uñates DR, et al. Versatile action of picomolar gradients of progesterone on different sperm subpopulations. *PLoS One.* 2014;9(3):e91181.
114. Gil PI, et al. Chemotactic response of frozen-thawed bovine spermatozoa towards follicular fluid. *Anim Reprod Sci.* 2008;108(1-2):236–46.
115. Jeon H, et al. Multi-dimensional-double-spiral (MDDS) inertial microfluidic platform for sperm isolation directly from the raw semen sample. *Sci Rep.* 2022;12(1):4212.
116. Vasilescu SA, et al. A microfluidic approach to rapid sperm recovery from heterogeneous cell suspensions. *Sci Rep.* 2021;11(1):7917.
117. De Toni L, et al. Heat sensing receptor TRPV1 is a mediator of thermotaxis in human spermatozoa. *PLoS One.* 2016;11(12):e0167622.
118. Gode F, et al. Comparison of microfluid sperm sorting chip and density gradient methods for use in intrauterine insemination cycles. *Fertil Steril.* 2019;112(5):842–8.e1.
119. Ozcan P, et al. Does the use of microfluidic sperm sorting for the sperm selection improve in vitro fertilization success rates in male factor infertility? *J Obstet Gynaecol Res.* 2021;47(1):382–8.
120. Yildiz K, Yuksel S. Use of microfluidic sperm extraction chips as an alternative method in patients with recurrent in vitro fertilisation failure. *J Assist Reprod Genet.* 2019;36(7):1423–9.
121. Yetkinel S, et al. Effects of the microfluidic chip technique in sperm selection for intracytoplasmic sperm injection for unexplained infertility: A prospective, randomized controlled trial. *J Assist Reprod Genet.* 2019;36(3):403–9.
122. Quinn MM, et al. Microfluidic preparation of spermatozoa for ICSI produces similar embryo quality to density-gradient centrifugation: A pragmatic, randomized controlled trial. *Hum Reprod.* 2022;37(7):1406–13.
123. Yalcinkaya Kalyan E, et al. Does a microfluidic chip for sperm sorting have a positive add-on effect on laboratory and clinical outcomes of intracytoplasmic sperm injection cycles? A sibling oocyte study. *Andrologia.* 2019;51(10):e13403.
124. Dubey A, et al. The influence of sperm morphology on pre-implantation genetic diagnosis cycles outcome. *Fertil Steril.* 2008;89(6):1665–9.
125. Watkins AJ, et al. Paternal programming of offspring health. *Early Hum Dev.* 2020;150:105185.
126. Bartoov B, et al. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. *J Androl.* 2002;23(1):1–8.
127. Teixeira DM, et al. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. *Cochrane Database Syst Rev.* 2020;2:CD010167.
128. Bartoov B, et al. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. *Fertil Steril.* 2003;80(6):1413–9.
129. de Almeida Ferreira Braga DP, et al. Sperm organelle morphologic abnormalities: Contributing factors and effects on intracytoplasmic sperm injection cycles outcomes. *Urology.* 2011;78(4):786–91.
130. Boitrelle F, et al. Small human sperm vacuoles observed under high magnification are pocket-like nuclear concavities linked to chromatin condensation failure. *Reprod Biomed Online.* 2013;27(2):201–11.
131. Boitrelle F, et al. The nature of human sperm head vacuoles: A systematic literature review. *Basic Clin Androl.* 2013;23:3.
132. Franco JG Jr., et al. Large nuclear vacuoles are indicative of abnormal chromatin packaging in human spermatozoa. *Int J Androl.* 2012;35(1):46–51.

133. Perdrix A, Rives N. Motile sperm organelle morphology examination (MSOME) and sperm head vacuoles: State of the art in 2013. *Hum Reprod Update*. 2013;19(5):527–41.
134. Setti AS, et al. Sperm morphological abnormalities visualised at high magnification predict embryonic development, from fertilisation to the blastocyst stage, in couples undergoing ICSI. *J Assist Reprod Genet*. 2014;31(11):1533–9.
135. Gao Y, et al. Motile sperm organelle morphology examination (MSOME) can predict outcomes of conventional in vitro fertilization: A prospective pilot diagnostic study. *Hum Fertil (Camb)*. 2015;18(4):258–64.
136. Greco E, et al. Sperm vacuoles negatively affect outcomes in intracytoplasmic morphologically selected sperm injection in terms of pregnancy, implantation, and live-birth rates. *Fertil Steril*. 2013;100(2):379–85.
137. Setti AS, et al. The prevalence of sperm with large nuclear vacuoles is a prognostic tool in the prediction of ICSI success. *J Assist Reprod Genet*. 2014;31(3):307–12.
138. Asali A, et al. The possibility of integrating motile sperm organelle morphology examination (MSOME) with intracytoplasmic morphologically-selected sperm injection (IMSI) when treating couples with unexplained infertility. *PLoS One*. 2020;15(5):e0232156.
139. Berkovitz A, et al. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. *Hum Reprod*. 2005;20(1):185–90.
140. Goswami G, et al. Can intracytoplasmic morphologically selected spermatozoa injection be used as first choice of treatment for severe male factor infertility patients? *J Hum Reprod Sci*. 2018;11(1):40–44.
141. Karabulut S, et al. Intracytoplasmic morphologically selected sperm injection, but for whom? *Zygote*. 2019;27(5):299–304.
142. Mangoli E, et al. IMSI procedure improves clinical outcomes and embryo morphokinetics in patients with different aetiologies of male infertility. *Andrologia*. 2019;51(8):e13340.
143. Mangoli E, et al. Association between early embryo morphokinetics plus transcript levels of sperm apoptotic genes and clinical outcomes in IMSI and ICSI cycles of male factor patients. *J Assist Reprod Genet*. 2020;37(10):2555–67.
144. Schachter-Safrai N, et al. Which semen analysis correlates with favorable intracytoplasmic morphologically selected sperm injection (IMSI) outcomes? *Eur J Obstet Gynecol Reprod Biol*. 2019;234:85–8.
145. Boediono A, et al. Morphokinetics of embryos after IMSI versus ICSI in couples with sub-optimal sperm quality: A time-lapse study. *Andrologia*. 2021;53(4):e14002.
146. Dieamant F, et al. Impact of intracytoplasmic morphologically selected sperm injection (IMSI) on birth defects: A systematic review and meta-analysis. *JBRA Assist Reprod*. 2021;25(3):466–72.
147. Duran-Retamal M, et al. Live birth and miscarriage rate following intracytoplasmic morphologically selected sperm injection vs intracytoplasmic sperm injection: An updated systematic review and meta-analysis. *Acta Obstet Gynecol Scand*. 2020;99(1):24–33.
148. Gatimel N, Parinaud J, Leandri RD. Intracytoplasmic morphologically selected sperm injection (IMSI) does not improve outcome in patients with two successive IVF-ICSI failures. *J Assist Reprod Genet*. 2016;33(3):349–55.
149. Teixeira DM, et al. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. *Cochrane Database Syst Rev*. 2013;(7):Cd010167.
150. Berkovitz A, et al. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. *Hum Reprod*. 2005;20(1):185–90.
151. Greco E, et al. Sperm vacuoles negatively affect outcomes in intracytoplasmic morphologically selected sperm injection in terms of pregnancy, implantation, and live-birth rates. *Fertil Steril*. 2013;100(2):379–85.
152. Gaspard O, et al. Impact of high magnification sperm selection on neonatal outcomes: A retrospective study. *J Assist Reprod Genet*. 2018;35(6):1113–21.
153. Prinosilova P, et al. Selectivity of hyaluronic acid binding for spermatozoa with normal Tygerberg strict morphology. *Reprod Biomed Online*. 2009;18(2):177–83.
154. Pregl Breznik B, Kovačič B, Vlaisavljević V. Are sperm DNA fragmentation, hyperactivation, and hyaluronan-binding ability predictive for fertilization and embryo development in in vitro fertilization and intracytoplasmic sperm injection? *Fertil Steril*. 2013;99(5):1233–41.
155. Esterhuizen AD, et al. Relationship between human spermatozoa-hyaluronan-binding assay, conventional semen parameters and fertilisation rates in intracytoplasmic spermatozoa injection. *Andrologia*. 2015;47(7):759–64.
156. Kovacs P, et al. The role of hyaluronic acid binding assay in choosing the fertilization method for patients undergoing IVF for unexplained infertility. *J Assist Reprod Genet*. 2011;28(1):49–54.
157. Choe SA, et al. Application of sperm selection using hyaluronic acid binding in intracytoplasmic sperm injection cycles: A sibling oocyte study. *J Korean Med Sci*. 2012;27(12):1569–73.
158. Scaruffi P, et al. Hyaluronic acid-sperm selection significantly improves the clinical outcome of couples with previous ICSI cycles failure. *Andrology*. 2022;10(4):677–85.
159. Kim SJ, et al. Effect of sperm selection using hyaluronan on fertilization and quality of cleavage-stage embryos in intracytoplasmic sperm injection (ICSI) cycles of couples with severe teratozoospermia. *Gynecol Endocrinol*. 2020;36(5):456–9.
160. Rezaei M, et al. Effect of sperm selection methods on ICSI outcomes in patients with oligoteratozoospermia. *Am J Clin Exp Urol*. 2021;9(2):170–6.
161. Miller D, et al. Physiological, hyaluronan-selected intracytoplasmic sperm injection for infertility treatment (HABSelect): A parallel, two-group, randomised trial. *Lancet*. 2019;393(10170):416–22.
162. West R, et al. Sperm selection with hyaluronic acid improved live birth outcomes among older couples and was connected to sperm DNA quality, potentially affecting all treatment outcomes. *Hum Reprod*. 2022;37(6):1106–25.
163. Novoselsky Persky M, et al. Conventional ICSI vs. physiological selection of spermatozoa for ICSI (picsi) in sibling oocytes. *Andrology*. 2021;9(3):873–7.
164. Jakab A, et al. Intracytoplasmic sperm injection: A novel selection method for sperm with normal frequency of chromosomal aneuploidies. *Fertil Steril*. 2005;84(6):1665–73.
165. Avalos-Durán G, et al. Physiological ICSI (PICSi) vs. conventional ICSI in couples with male factor: A systematic review. *JBRA Assist Reprod*. 2018;22(2):139–47.
166. Beck-Fruchter R, Shalev E, Weiss A. Clinical benefit using sperm hyaluronic acid binding technique in ICSI cycles: A systematic review and meta-analysis. *Reprod Biomed Online*. 2016;32(3):286–98.
167. Giuliani V, et al. Expression of gp20, a human sperm antigen of epididymal origin, is reduced in spermatozoa from subfertile men. *Mol Reprod Dev*. 2004;69(2):235–40.
168. Ainsworth C, Nixon B, Aitken RJ. Development of a novel electrophoretic system for the isolation of human spermatozoa. *Hum Reprod*. 2005;20(8):2261–70.
169. Gianaroli L, et al. Sperm head's birefringence: A new criterion for sperm selection. *Fertil Steril*. 2008;90(1):104–12.
170. Gianaroli L, et al. Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection. *Fertil Steril*. 2010;93(3):807–13.
171. Mallidis C, et al. Raman microspectroscopy: Shining a new light on reproductive medicine. *Hum Reprod Update*. 2013;20(3):403–14.

8

SPERM CHROMATIN ASSESSMENT

Ashok Agarwal and Rakesh Sharma

Introduction

Semen analysis is used routinely to evaluate infertile men. Attempts to introduce quality control within and between laboratories have highlighted the subjectivity and variability of traditional semen parameters. A significant overlap in sperm concentration, motility, and morphology between fertile and infertile men has been demonstrated [1]. In addition, standard measurements may not reveal subtle sperm defects such as DNA damage, and these defects can affect fertility. New markers are needed to better discriminate infertile men from fertile ones, predict pregnancy outcomes in the female partner, and calculate the risk of adverse reproductive events. In this context, sperm chromatin abnormalities have been studied extensively in past decades as a cause of male infertility [2, 3]. Focus on the genomic integrity of the male gamete has been intensified due to growing concerns about transmission of damaged DNA through assisted reproduction technologies (ARTs), especially intracytoplasmic sperm injection (ICSI). It is a particular concern if the amount of sperm DNA damage exceeds the repair capacity of oocytes. There are concerns related to potential chromosomal abnormalities, congenital malformations, and developmental abnormalities in ICSI-born progeny [4, 5].

Accumulating evidence suggests that a negative relationship exists between disturbances in the organization of the genomic material in sperm nuclei and the fertility potential of spermatozoa, whether *in vivo* or *in vitro* [6–14]. Abnormalities in the male genome characterized by damaged sperm DNA may be indicative of male subfertility regardless of normal semen parameters [15, 16]. Sperm chromatin structure evaluation is an independent measure of sperm quality that provides good diagnostic and prognostic capabilities. Therefore, it may be considered a reliable predictor of a couple's inability to become pregnant [17]. This may have an impact on the offspring, resulting in trans-generational infertility [18].

Poor intrauterine insemination (IUI) outcomes have been reported with elevated levels of sperm DNA fragmentation (SDF) in the semen [12]. A number of studies has shown the deleterious effect of SDF on IVF and ICSI outcomes, such as clinical pregnancies, miscarriages, and recurrent pregnancy loss [19–23]. These studies provide supporting evidence of the clinical utility of SDF testing in situations of repeat ART failure. SDF testing has emerged as a simple tool complementary to the conventional semen analysis that may enable clinicians to better manage infertile couples. The reproductive outcome data (live birth rate) is still limited [24–26] and American Society for Reproductive Medicine (ASRM), the American Urological Association (AUA), the European Association of Urology (EAU), and the National Institute of Clinical Excellence (NICE) still do not officially recommend SDF tests as an adjunct to male infertility assessment [27–29]. The clinical value of SDF testing has been confirmed in recent studies [30–36]. The guidelines of the Society for

Translational Medicine recommend SDF testing as it may influence the clinical management of infertile patients with varicocele, since men with high-grade varicocele often present with normal semen parameters, while impaired semen parameters often correspond with low-grade varicocele patients with high levels of sperm DNA damage [37–40]. The WHO sixth edition (2021) now includes SDF testing [41].

Many techniques have been described to evaluate the sperm chromatin status. In this chapter, we describe the normal sperm chromatin architecture and the causative factors leading to its aberrations. We also provide the rationale for sperm chromatin assessment and discuss the different methods used to analyse sperm DNA integrity.

Human sperm chromatin structure

In many mammals, spermatogenesis leads to the production of highly homogenous spermatozoa. For example, more than 95% of the nucleoprotein in mouse sperm nuclei is composed of protamines [42]. This allows mature sperm nuclei to adopt a volume 40 times less than that of normal somatic nuclei [43]. The final, highly compact packaging of the primary sperm DNA filament is produced by DNA–protamine complexes. Contrary to nucleosomal organization in somatic cells, which is provided by histones, these DNA–protamine complexes approach the physical limits of molecular compaction [44, 45]. Human sperm nuclei, on the other hand, contain considerably fewer protamines (around 85%) than sperm nuclei of the bull, stallion, hamster, and mouse [46, 47]. Mature human spermatozoa contain some levels of nucleosomes, which are believed to be necessary for organizing higher-order genomic structure through interactions with the nuclear matrix. These regions are non-randomly distributed throughout the sperm genome [48]. Human sperm chromatin is therefore less regularly compacted and frequently contains DNA strand breaks [49].

To achieve this uniquely condensed state, sperm DNA must be organized in a specific manner that differs substantially from that of somatic cells [50]. The fundamental packaging unit of mammalian sperm chromatin is a toroid containing 50–60 kilobases of DNA. Individual toroids represent the DNA loop domains that are highly condensed by protamines and fixed at the nuclear matrix. Toroids are cross-linked by disulphide bonds formed by oxidation of sulphhydryl groups of cysteine present in the protamines [50]. Thus, each chromosome represents a garland of toroids, while all 23 chromosomes are clustered by centromeres into a compact chromocenter positioned well inside the nucleus; the telomere ends are united into dimers exposed to the nuclear periphery [51, 52]. This condensed, insoluble, and highly organized nature of sperm chromatin acts to protect the genetic integrity during transport of the paternal genome through the male and female reproductive tracts. It also ensures that the paternal DNA is delivered in the form that sterically allows the proper fusion of

two gametic genomes and enables the developing embryo to correctly express the genetic information [52–53].

In comparison with other species [54], human sperm chromatin packaging is exceptionally variable both within and between men. This variability has been mostly attributed to its basic protein component. The retention of 15% histones, which are less basic than protamines, leads to the formation of a less compact chromatin structure [47]. Moreover, in contrast to the bull, cat, boar, and ram—whose spermatozoa contain only one type of protamine (P1)—human and mouse spermatozoa contain a second type of protamine called P2, which is deficient in cysteine residues [55]. Consequently, the disulphide cross-linking that is responsible for more stable packaging is diminished in human sperm as compared with species containing P1 alone [56]. The relative proportion of P1 to P2 is regulated at approximately 1:1 ratio at both mRNA and protein levels [57, 58]. This protamine ratio is unaltered in fertile men, but altered P1/P2 ratios and the absence of P2 are associated with male fertility problems [59–65]. Aberrant P1/P2 ratio is also associated with low fertilization rate and poor embryo quality [66]. A recent study reported that poor sperm protamination was associated with the development of low-quality embryos after *in vitro* fertilization [67]. The P1/P2 ratio has been shown to correlate with SDF, and significant differences were detected between fertile and infertile men [68]. The reference range reported for P1/P2 in a fertile, normozoospermic population ranges from 0.54 to 1.43. Such a wide range of P1/P2 shows that abnormal protamination can be an indicator of other disturbances that occur during spermatogenesis that can cause infertility [69]. Altered mRNA P1/P2 ratio has been shown to be

a valuable indicator of sperm maturity and fertilization ability and, in these cases, patients can benefit from the use of testicular sperm [64].

Origin of sperm chromatin abnormalities

The susceptibility of male germ cells to DNA damage stems partly from the down-regulation of DNA repair systems during late spermatogenesis. In addition, the cellular machinery that allows these cells to undergo complete apoptosis is progressively lost during spermatogenesis. As a result, the advanced stages of germ cell differentiation cannot be deleted, even though they may have proceeded some way down the apoptotic pathway. As a consequence, the ejaculated gamete may exhibit genetic damage. Such DNA damage will be carried into the zygote by the fertilizing spermatozoon and must be then repaired, preferably prior to the first cleavage division. Several studies have shown that oocytes and early embryos can repair sperm DNA damage [70, 71]. Consequently, the biological effect of abnormal sperm chromatin structure depends on the combined effects of sperm chromatin damage and the capacity of the oocyte to repair it. Any errors that may occur during this post-fertilization period of DNA repair have the potential to create mutations that can affect fetal development and, ultimately, the health of the child [18, 72]. DNA damage can be a result of mismatch of bases, loss of base (abasic site), base modifications, DNA adducts and cross link, pyrimidine dimers and single-strand breaks (SSB), and double-strand breaks (DSB) [31] (Figure 8.1). Any of these alterations can induce SDF and compromise natural conception or ART outcomes.

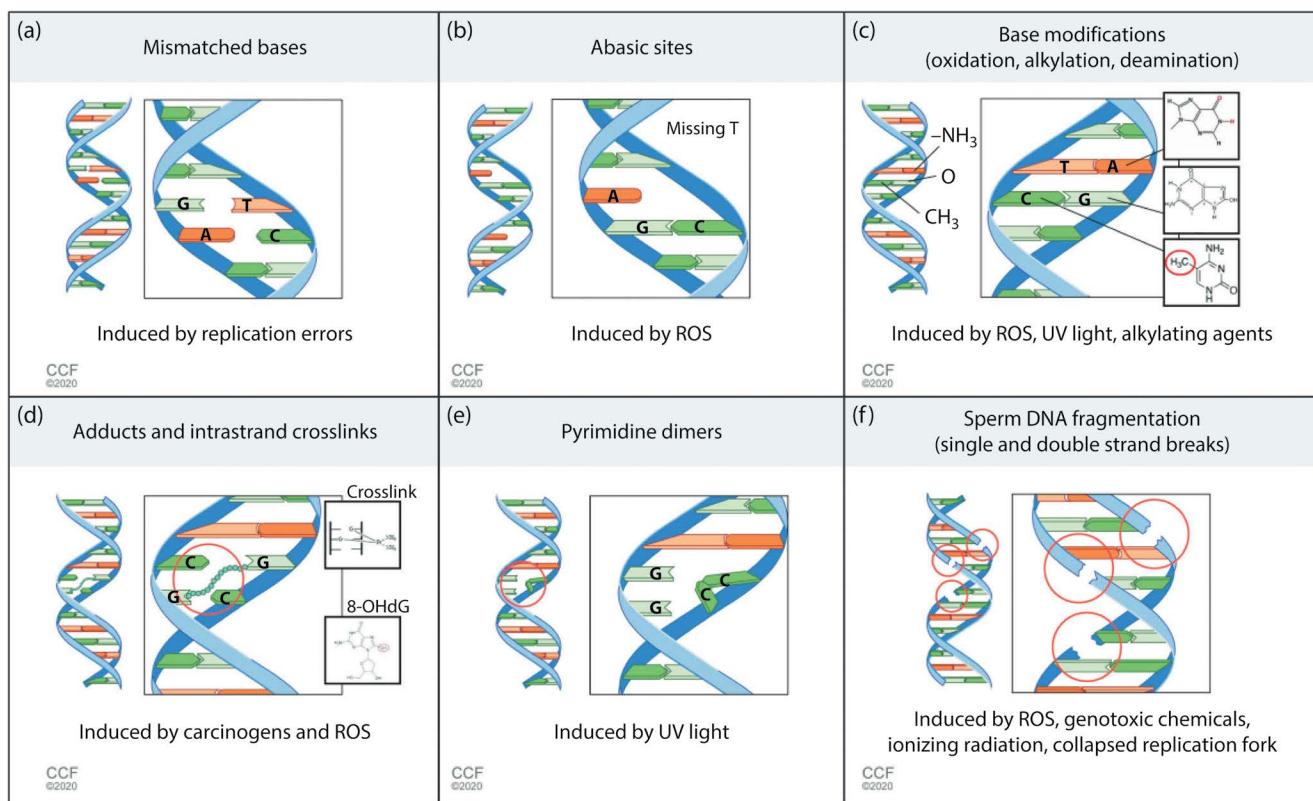


FIGURE 8.1 Different types of DNA damage. (a) Mismatched bases, (b) abasic sites, (c) base modifications (oxidation, alkylation, deamination), (d) adducts and intrastrand cross links, (e) pyrimidine dimers, and (f) single- and double-strand fragmentation. Abbreviations: ROS, reactive oxygen species; UV, ultraviolet.

The exact mechanisms by which chromatin abnormalities/DNA damage arise in human spermatozoa are not completely understood. Three main theories have been proposed: defective sperm chromatin packaging, abortive apoptosis, and oxidative stress (OS) [73]. Deficiencies in recombination may also play a role.

Defective sperm chromatin packaging

Stage-specific introduction of transient DNA strand breaks during spermiogenesis has been described [73–76]. DNA breaks have been found in round and elongating spermatids. Such breaks are necessary for transient relief of torsional stress. During maturation, the nucleosome histone cores in elongating spermatids are cast off and replaced with transitional proteins and protamines [73, 77, 78]. Thus, chromatin repackaging includes a sensitive step necessitating endogenous nuclease activity, which is evidently fulfilled by coordinated loosening of the chromatin by histone hyperacetylation and by topoisomerase II, which can create and ligate breaks [77, 78]. Although there is little evidence to suggest that spermatid maturation-associated DNA breaks are fully ligated, unrepaired DNA breaks are not allowed [79].

Ligation of DNA breaks is necessary not only to preserve the integrity of the primary DNA structure but also for reassembly of the important unit of genome expression—the DNA loop domain. Interaction of sperm DNA with protamines results in the coiling of sperm DNA into toroidal subunits known as doughnut loops [80]. If these temporary breaks are not repaired because of excessive topoisomerase II activity or a deficiency of topoisomerase II inhibitors [81, 82], then DNA fragmentation in ejaculated spermatozoa may result. Similarly, if appropriate disulphide bridge formation does not occur because of inadequate oxidation of thiols during epididymal transit, the DNA will be more vulnerable to damage caused by suboptimal compaction. Recent studies have postulated the hypothesis that large nuclear vacuoles could be an indicator of abnormal chromatin packaging [83, 84].

Further, the ratio of P1 to P2 maintained by P2 precursor (pre-P2) has a crucial role in sperm fertilization. Abnormal sperm morphogenesis with reduced motility can also result due to defective pre-P2 mRNA translation [69, 85–88].

Abortive apoptosis

The incidence of apoptosis in ejaculated sperm is still a contentious issue. Until recently, the inability of a mature spermatozoon to synthesize new proteins was believed to make it impossible for such cells to respond to any of the signals that lead to the programmed death cascade. However, a number of recent observations have raised the possibility that abortive apoptosis may contribute to DNA damage in human spermatozoa: (1) the detection of Fas on ejaculated spermatozoa [89]; (2) the high proportion of spermatozoa with potentially apoptotic mitochondria [90]; and (3) the finding that potential mediators of apoptosis, including endonuclease activity, are present in spermatozoa [91]. It has been postulated that OS can interfere with sperm chromatin remodelling. Cells with altered chromatin structure can enter the apoptotic pathway, which is characterized by loss of motility, caspase activation, phosphatidylserine externalization, and the activation of reactive oxygen species (ROS) generation by the mitochondria. ROS causes lipid peroxidation and oxidative DNA damage, which, in turn, leads to DNA fragmentation and eventually cell death [92].

It has been suggested that an early apoptotic pathway, initiated in spermatogonia and spermatocytes, is mediated by Fas protein. Fas is a type I membrane protein that belongs to the tumour necrosis factor–nerve growth factor receptor family [93]. It has been shown that Sertoli cells express Fas ligand, which by binding to Fas leads to cell death via apoptosis [93]. This in turn limits the size of the germ cell population to a number that Sertoli cells can support [94]. Ligation of Fas ligand to Fas in the cellular membrane triggers the activation of caspases and therefore this pathway is also characterized as a caspase-induced apoptosis [95].

Men exhibiting deficiencies in their semen profile often possess a large number of spermatozoa that bear Fas. This fact prompts the suggestion that these dysfunctional cells are the product of an incomplete apoptotic cascade [49]. However, a contribution of aborted apoptosis in the DNA damage seen in the ejaculated spermatozoa is doubtful in cases where this process is initiated at the early stages of spermatogenesis. This is because at the stage of DNA fragmentation, apoptosis is an irreversible process [96], and these cells should be digested by Sertoli cells and removed from the pool of ejaculated sperm. Some studies have not found correlations between DNA damage and Fas expression [97] or, in contrast, have not revealed ultrastructural evidence for the association of apoptosis with DNA damage in sperm [98]. Alternatively, if the apoptotic cascade is initiated at the round spermatid phase, where transcription (and mitochondria) is still active, abortive apoptosis might be an origin of the DNA breaks. A Bcl2 anti-apoptotic family gene member called Bclw has been shown to suppress apoptosis in elongating spermatids [99]. Although many apoptotic biomarkers have been found in the mature male gamete, particularly in infertile men, their definitive association with DNA fragmentation remains elusive [100–108].

Oxidative stress

Normal levels of ROS play an important physiological role, modulating gene and protein activities that are vital for sperm proliferation, differentiation, and function. In semen, the amount of ROS generation is controlled by seminal antioxidants that ensure a balance between ROS and antioxidant capacity. Any imbalance that occurs either by high ROS production or low antioxidant levels leads to OS [109–114]. The human spermatozoon is highly susceptible to OS [115]. This process induces peroxidative damage in the sperm plasma membrane and DNA fragmentation. A number of pro-inflammatory cytokines at physiological levels are responsible for the lipid peroxidation of sperm membrane, which is considered important for the fecundation capacity of the spermatozoa. However, OS may lead to abnormal production of certain interleukin/cytokines such as IL-8 and TNF- α , either alone or in combination with any infection, which may be able to drive the lipid peroxidation to a level that can affect the sperm fertilizing capacity [116]. Such stress may arise from a variety of sources. Morphologically abnormal spermatozoa (with residual cytoplasm, in particular) and leukocytes are the main sources of excessive ROS generation in semen [109]. Also, a lack of antioxidant protection and the presence of redox cycling xenobiotics may be the cause of OS. Antioxidant supplementation in these infertile men has been reported to be beneficial [117]. Whenever levels of OS in the male germ line are high, the peroxidation of unsaturated fatty acids in the sperm plasma membrane leads to the depressed fertilization rates associated with DNA damage [18, 37, 39, 40, 118].

Single- and double-strand DNA breaks

DNA fragmentation may be due to single-strand breaks (SSBs) or double-strand breaks (DSBs) (Figure 8.2). Single-strand breaks (SSBs) are a result of abortive topoisomerase or DNA ligase activity adjacent to a lesion and can be easily repaired [119]. The most common SSBs are base and sugar modifications and SSBs following oxidation, alkylation, deamination, and spontaneous hydrolysis. OS, lipid peroxidation, and protein alteration may also lead to SSBs (Figure 8.2a). When these lesions are not repaired, they can compromise the integrity of the genome. Endogenous sources during the DNA replication process, collapsed replication forks, or increased levels of free radicals all result in increased DSBs (Figure 8.2b).

In addition, exogenous sources such as ionizing radiation, genotoxic chemicals, and radiomimetic drugs can also lead to DSBs. Both SSBs and DSBs can affect the overall fertility and reproductive outcomes. Higher levels of SSBs are inversely related to the natural pregnancy outcome [120]. DSBs are more damaging to the genomic integrity and have been associated with recurrent miscarriages in couples without a female factor [120].

Deficiencies in recombination

Meiotic crossing-over is associated with the genetically programmed introduction of DNA double-strand breaks (DSBs) by specific nucleases of the SPO11 family [121]. These DNA DSBs should be ligated until the end of meiosis I. Normally, a recombination checkpoint in meiotic prophase does not allow meiotic division I to proceed until DNA is fully repaired or defective spermatocytes are ablated [121]. A defective checkpoint may lead to persistent SDF in ejaculated spermatozoa, although direct data for this hypothesis in humans is lacking.

The processes leading to DNA damage in ejaculated sperm are inter-related. For example, a defective spermatid protamination and disulphide bridge formation caused by inadequate oxidation of thiols during epididymal transit, resulting in diminished sperm chromatin packaging, makes sperm cells more vulnerable to ROS-induced DNA fragmentation. A two-step hypothesis has been proposed, suggesting that OS acts on poorly protaminated cells that are generated as a result of defective spermiogenesis [122].

Contributing factors

Advanced paternal age, smoking, obesity, radiofrequency, electromagnetic radiation, and xenobiotics are the common factors attributed to sperm DNA damage [30, 37, 123–127]. Advancing age has been associated with an increased percentage of ejaculated spermatozoa with DNA damage [11, 128–131]. Young men with cancer typically have poor semen quality and sperm DNA damage even before starting the therapy. Further damage from radiation or chemotherapy is dependent on both the duration and dose of radiation [132, 133]. Spermatogenesis may not occur months to years after therapy, but evidence of sperm DNA damage often persists beyond that period [134, 135]. Data on men with testicular cancer showed that radiation therapy induced transient sperm DNA damage and that this damage was present three to five years later, but three or more cycles of chemotherapy, in turn, decreased the percentage of sperm with DNA damage [135].

Cigarette smoking is associated with a decrease in sperm count and motility and an increase in abnormal sperm forms and sperm DNA damage [126]. It is suggested that smoking increases production of leukocyte-derived ROS; the OS may be the underlying reason why sperm DNA from smokers contains more strand breaks than that from non-smokers [136]. Also, genital tract infections and inflammation result in leukocytospermia and have been associated with OS and subsequent sperm DNA damage [137]. Exposure to pesticides (organophosphates), persistent organochlorine pollutants, and air pollution have also been associated with sperm DNA damage [11, 138]. Varicocele has been associated with seminal OS and sperm DNA damage. Clinical varicoceles cause both single-strand and double-strand breaks. In addition, in normozoospermic men, clinically significant varicoceles can also cause sperm DNA damage without alterations in the conventional semen parameters result in sperm [39, 139–148].

Various contributing factors for sperm DNA damage are shown in Figure 8.3. These may originate from defective sperm maturation, abortive apoptosis, and oxidative stress, along with other clinical and environmental risk factors [31].

Sperm DNA integrity has been shown to improve after varicocele repair [40, 149–154].

A deficiency in gonadotropin hormones such as follicle stimulating hormone (FSH) can cause sperm chromatin defects. FSH

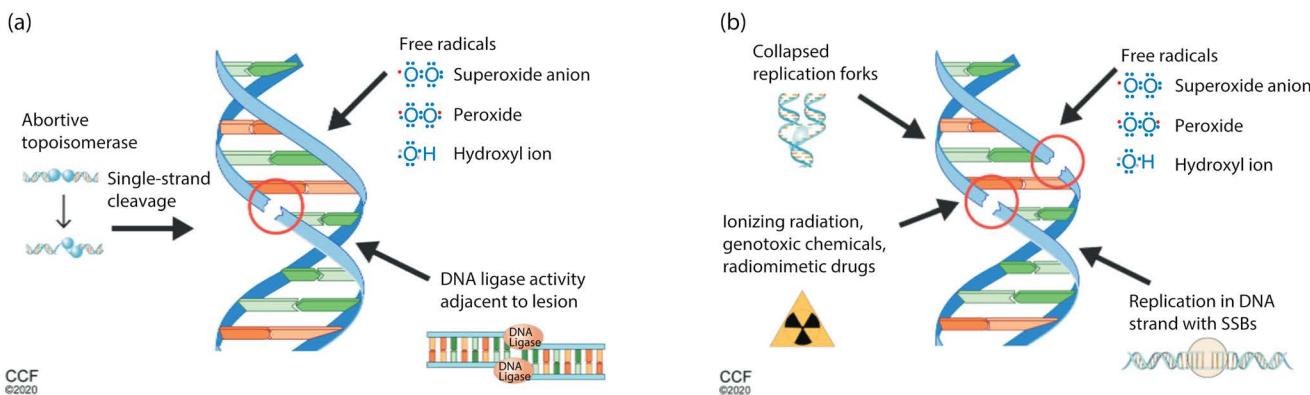


FIGURE 8.2 (a) Single-strand breaks in DNA are a result of abortive topoisomerase, free radicals, and DNA ligase activity adjacent to lesion. (b) Double-strand DNA breaks are caused by free radicals; collapsed replication forks; replication in DNA strand with single-strand breaks; and ionizing radiation, genotoxic chemicals, and radiomimetic drugs.

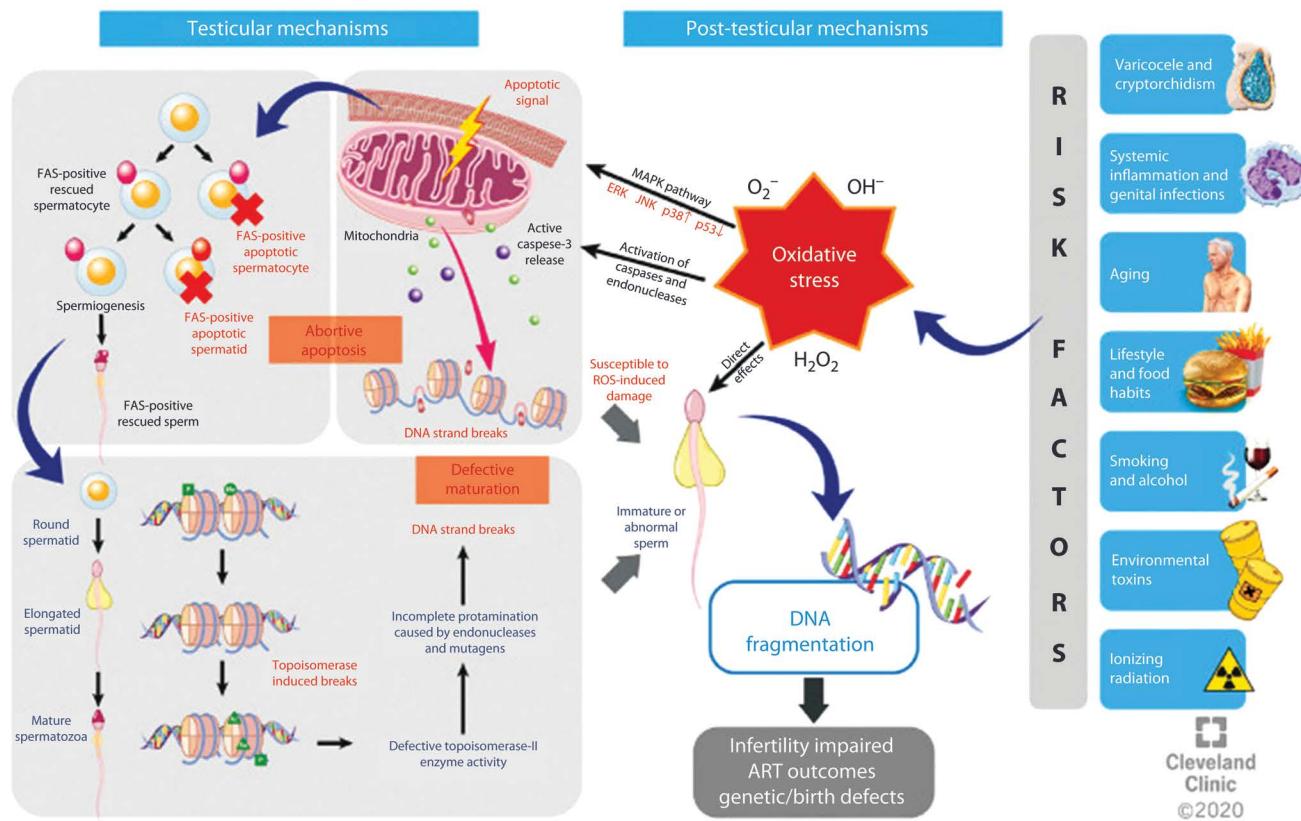


FIGURE 8.3 Overview of the origins of sperm DNA fragmentation (SDF). Underlying mechanisms such as defective maturation, abortive apoptosis, and oxidative stress can result in SDF. Clinical (age, infection, cancer, hormonal imbalances, obesity, diabetes) and environmental (heat exposure, environmental toxins, radiation, smoking, drug abuse, diet) risk factors also result in SDF. Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-JUN N-terminal kinase; ROS, reactive oxygen species; ART, assisted reproductive techniques.

receptor-knockout mice have been found to have higher levels of DNA damage in sperm [155]. Febrile illness has been shown to cause an increase in the histone/protamine ratio and DNA damage in ejaculated sperm [156]. Direct mild testicular and epididymal hyperthermia has also been shown to cause these effects [157]. Finally, sperm preparation techniques involving repeated high-speed centrifugation and the isolation of spermatozoa from the seminal plasma, which is a protective antioxidant environment, may contribute to increased sperm DNA damage via mechanisms that are mediated by the enhanced generation of ROS [14].

Indications for sperm chromatin assessment

Evaluating sperm chromatin can be challenging for several reasons: it can be difficult to link the results of chromatin integrity tests to known physiological mechanisms; the role that sperm chromatin structure assessment plays in clinical practice (especially in ART) is still controversial; and there is no one standardized method for measuring sperm chromatin integrity [17, 39, 158–162]. On the other hand, sperm chromatin structure is complex, and several methods may be necessary in order to assess this. In addition, a number of confounding factors can complicate the interpretation of the results, including heterogeneity in the sperm population and the fact that not all DNA damage is lethal (most

DNA contains non-coding regions or introns, and oocytes can repair sperm DNA damage). Nevertheless, at the present time, it is clear that sperm chromatin assessment provides good diagnostic and prognostic capabilities for fertility/infertility [27–29].

It must be stressed that among all methods employed for sperm chromatin assessment, clinical thresholds so far have been demonstrated only for the sperm chromatin structure assay (SCSA) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay, and these thresholds have been confirmed by different laboratories for SCSA only. However, a recent study published a detailed step-by-step approach for measuring SDF by TUNEL using a benchtop flow cytometer, which is user friendly and facilitates data interpretation [163]. A cross-sectional survey across 19 countries showed that 30.6% of SDF measurements are done using TUNEL and SCSA, whereas 20.4% and 6.1% use sperm chromatin dispersion (SCD) and single-cell gel electrophoresis (Comet), respectively [162]. Also, the reported biological variability of sperm DNA damage within men over time should be considered, although it is more stable than standard semen parameters [164–166]. Indications for sperm DNA evaluation include male infertility diagnosis (varicocele, idiopathic infertility) recurrent pregnancy loss, unexplained infertility, use of ARTs, and follow-up after oncological treatment such as radiotherapy or chemotherapy.

Diagnosis of male infertility

Although a spermatozoon with damaged DNA can fertilize an egg, future embryonic growth is compromised, which may ultimately lead to miscarriage or childhood deformities. Many studies have shown, using a variety of techniques, significant differences in sperm DNA damage levels between fertile and infertile men [167–171]. Moreover, spermatozoa from infertile patients are generally more susceptible to the effects of DNA-damaging agents such as H₂O₂, smoking, obesity, and radiation [172]. The probability of fertilization *in vivo* reduces drastically if the proportion of sperm cells with DNA damage exceeds 30% as detected by the SCSA [17, 173] or 20% as detected by TUNEL [174]. However, the latest commentary on the utilization of SDF testing in fertility outcomes suggests avoiding this test. The debate argues that several couples have become pregnant even though the threshold of DNA damage was higher than what we consider normal; in addition, some studies failed to find any difference in outcome in men who differ in SDF levels [175]. In continuation of such investigations, some people support the diagnostic value of sperm DNA integrity and suggest that it may be considered an objective marker of sperm function that serves as a significant prognostic factor for male infertility [7, 17, 29]. A significant increase in SCSA-defined DNA damage in sperm from infertile men with normal sperm parameters has been demonstrated [170], indicating that analysis of sperm DNA damage may reveal a hidden sperm abnormality in infertile men classified with idiopathic infertility based on apparently normal standard semen parameters.

Assisted reproduction technologies

The probability of fertilization by IUI or IVF is reduced in cases where the proportion of sperm cells with DNA damage exceeds 30% by means of SCSA [12, 176, 177]. As described in the previous section, the controversy as to whether sperm DNA damage negatively affects the results of IVF and ICSI has yet to be resolved [178–180]. Although no association between sperm DNA damage and IVF/ICSI outcome has been demonstrated in some studies [181], most show a significant negative correlation between sperm DNA damage and embryo quality in IVF cycles [23, 182], blastocyst development following IVF [183], and fertilization rates following IVF [179, 184] and ICSI, even though sperm DNA damage may not necessarily preclude fertilization and pronucleus formation during ICSI [185, 186]. In addition to other studies, two meta-analyses concluded that sperm DNA damage is predictive for reduced pregnancy success using routine IVF but has no significant effect on ICSI outcome [9, 20, 23, 25, 159, 187, 188]. Thus, assessment of sperm chromatin may help predict the success rates of IUI and IVF. It has been also suggested that in patients with a high proportion of DNA-damaged sperm who are seeking to use ART, ICSI should be the method of choice [12, 189–191].

Embryonic loss

Data on miscarriages as a possible consequence of sperm DNA damage are rather scarce. It has been shown that the proportion of sperm with DNA damage is significantly higher in men from couples with recurrent pregnancy loss than in the general population or fertile donors [20, 22, 160, 192]. It has also been reported that 39% of miscarriages could be predicted using a combination of selected cut-off values for percentage spermatozoa with denatured (likely fragmented) DNA and/or abnormal chromatin

packaging as assessed by SCSA [17]. The percentage of spontaneous abortions following IVF/ICSI was increased when sperm with high levels of DNA damage were used [19, 20, 192, 193], which highlights the need to assess sperm DNA damage in order to predict possible future miscarriage [22].

Cancer patients

Sperm DNA evaluation in patients with cancer requires special attention when future fertility and the health of the baby are considered [194]. The stressful micro-environment that develops during cancer can cause OS, which indirectly can damage sperm DNA. Patients with cancer are often referred to sperm banks before chemotherapy, radiation therapy, or surgery is initiated. Data suggest there is compromised semen quality, including DNA integrity, before the commencement of treatment [195, 196] and increased chromosomal aneuploidy after chemotherapy [197–201]. The extent of DNA damage may help to determine how semen should be cryopreserved before therapy begins. Specimens with high sperm concentrations and motility and low levels of DNA damage should be preserved in relatively large aliquots that are suitable for IUI [202]. If a single specimen of good quality is available, then it should be preserved in multiple small aliquots suitable for IVF or ICSI [198, 203, 204].

Methods used in the evaluation of sperm chromatin/DNA integrity

Different methods can be used to evaluate the status of the sperm chromatin/DNA for the presence of abnormalities or simply immaturity (Table 8.1). These methods include simple staining techniques such as the acidic aniline blue (AAB) and basic toluidine blue (TB), fluorescent staining techniques such as the sperm chromatin dispersion (SCD) test, chromomycin A₃ (CMA₃), DNA breakage detection–fluorescence *in situ* hybridization (DBD–FISH), *in situ* nick translation (NT), and flow cytometric-based SCSA. Some techniques employ more than one method for the analysis of their results. Examples of these include the acridine orange (AO) and TUNEL assays. Other methods less frequently used include measurement of 8-hydroxy-2-deoxyguanosine (8-OHdG) by high-performance liquid chromatography (HPLC).

AAB staining

Principle

Aniline blue is an acidic dye that has more binding affinity with the proteins in decondensed or loose chromatin due to the residual histones. AAB staining differentiates between lysine-rich histones and arginine/cysteine-rich protamines. This technique provides a specific positive reaction for lysine and reveals differences in the basic nuclear protein composition of ejaculated human spermatozoa. Histone-rich nuclei of immature spermatozoa are rich in lysine and will consequently take up the blue stain. On the other hand, protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not take up the stain [219].

Technique

Slides are prepared by smearing 5 µL of either a raw or washed semen sample, which is air-dried and fixed for 30 minutes in 3% glutaraldehyde in phosphate-buffered saline (PBS). The fixed smear is dried and stained in 5% aqueous aniline blue solution (pH 3.5) for five minutes. The staining characteristics depict the

TABLE 8.1 Various Methods for Assessing Sperm Chromatin Abnormalities

Assay	Parameter	Method of Analysis
Acidic aniline blue [205]	Nuclear maturity (DNA protein composition)	Optical microscopy
Toluidine blue staining [206]	Nuclear maturity (DNA protein composition)	Optical microscopy
Chromomycin A ₃ [207]	Nuclear maturity (DNA protein composition)	Fluorescence microscopy
DNA breakage detection–fluorescence <i>in situ</i> hybridization [208]	DNA fragmentation (ssDNA)	Fluorescence microscopy
<i>In situ</i> nick translation [209]	DNA fragmentation (ssDNA)	Fluorescence microscopy Flow cytometry
Acridine orange [210]	DNA denaturation (acid)	Fluorescence microscopy Flow cytometry
Sperm chromatin dispersion [119, 211]	DNA fragmentation	Fluorescence microscopy
Comet (neutral) [148, 212]	DNA fragmentation (dsDNA)	Fluorescence microscopy
Comet (alkaline) [22, 148, 213]	DNA fragmentation (ssDNA/dsDNA)	
TUNEL [163, 164, 174, 214–216]	DNA fragmentation	Fluorescence microscopy Flow cytometry Flow cytometry
Sperm chromatin structure assay [17]	DNA denaturation (acid/heat)	Flow cytometry
8-OHdG measurement [217, 218]	8-OHdG	High-performance lipid chromatography

Abbreviations: 8-OHdG, 8-hydroxy-2-deoxyguanosine; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

status of nuclear maturity. Sperm heads containing immature nuclear chromatin stain blue and those with mature nuclei do not. A total of 200 spermatozoa per slide are counted using bright field microscopy, and the percentage of spermatozoa stained with aniline blue is determined [205].

Modification of the AAB assay with eosin

One of the limitations of AAB staining is poor visualization of unstained sperm cells under ordinary light microscopy. To overcome this issue, counterstaining using eosin-Y is recommended. Sperm smears are fixed in 4% formalin solution for five minutes and rinsed in water. Slides are stained in 5% aniline blue prepared in 4% acetic acid (pH 3.5) solution for five minutes, rinsed in water, and counterstained in 0.5% eosin for one minute followed by rinsing and air drying [220].

Clinical significance

AAB staining has shown a linkage between chromatin immaturity and male infertility. In patients with varicocele, unilateral cryptorchidism, and idiopathic infertility, high sperm nuclear

instability with a higher number of AAB-stained spermatozoa was observed [221]. However, the correlation between the percentage of aniline blue-stained spermatozoa and other sperm parameters remains controversial. AAB-stained spermatozoa showed normal conventional parameters such as count, motility, and morphology [222]. Immature sperm chromatin may or may not correlate with asthenozoospermic samples and abnormal morphology patterns [219]. Most important is the finding that chromatin condensation as visualized by aniline blue staining is a good predictor for IVF outcome, although it cannot determine the fertilization potential and the cleavage and pregnancy rates following ICSI [220]. Evaluation of sperm chromatin using AAB staining could be considered as one of the complementary tests of semen analysis for assessment of male factor infertility [223, 224]. Counterstaining with eosin can facilitate interpretation of sperm chromatin integrity [220].

Advantages and limitations

The AAB technique is simple and inexpensive and requires only bright field microscopy for analysis. The only drawback is the heterogeneous slide staining.

TB staining

Principle

Toluidine Blue (TB), or tolonium chloride, is a basic thiazine metachromatic dye that selectively binds the acidic components of the tissue. It partially dissolves in water and alcohol. Alternatively known as methylamine or aminotoluene, the dye represents three isoforms: ortho-toluidine, para-toluidine, and meta-toluidine. It has high binding affinity for phosphate residues of sperm DNA in immature nuclei and provides a metachromatic shift from light blue to a purple–violet colour [225]. This stain is a sensitive structural probe for DNA structure and packaging.

Technique

TB staining follows the principle of metachromasia in which a dye can absorb light at different wavelengths and can change colour without changing chemical structure. Sperm smears are air-dried, fixed in freshly made 96% ethanol–acetone (1:1) at 4°C for at least 30 minutes, hydrolysed in 0.1 N HCl at 4°C for five minutes, and rinsed three times in distilled water for two minutes each. Smears are stained with 0.05% TB for five minutes. The staining buffer consists of 50% citrate phosphate (McIlvain buffer, pH 3.5). Permanent preparations are dehydrated in tertiary butanol twice for three minutes each at 37°C and in xylene twice for three minutes each; the preparations are embedded in DPX (a mixture of distyrene, a plasticizer, and xylene). Sperm heads with good chromatin integrity stain light blue and those of compromised integrity stain violet (purple) [226]. The results of the TB test are visualized using light microscopy. Based on the different optical densities of cells stained with TB, the image analysis cytometry test is elaborated (Figure 8.4a–c) [205].

Clinical significance

TB staining may be considered a fairly reliable method for assessing sperm chromatin. Abnormal nuclei (purple–violet sperm heads) have been shown to be correlated with counts of red–orange sperm heads as revealed by the AO method [225]. Significant correlations between the results of the TB, SCSA, and TUNEL tests have been demonstrated [226]. Clinical applicability of the TB test for male fertility potential assessment has also been demonstrated, with specificity for infertility diagnosis as high as

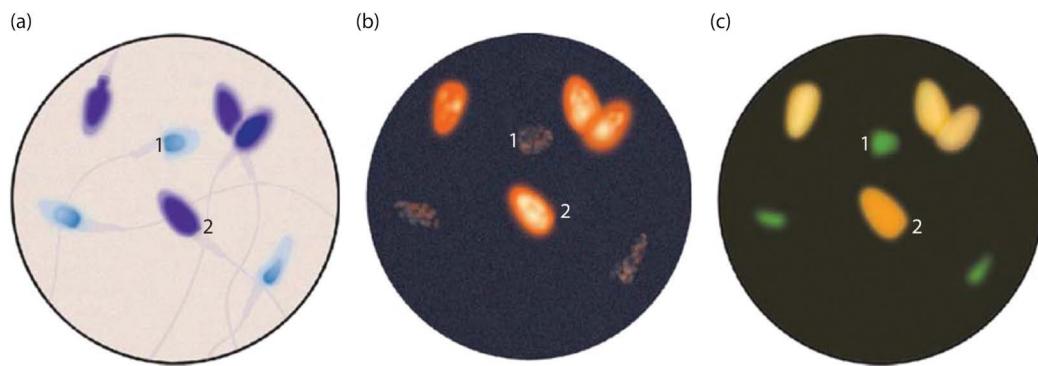


FIGURE 8.4 (a) Human ejaculate stained with toluidine blue: (1) sperm heads with normal chromatin conformation are light blue; (2) sperm heads with abnormal chromatin conformation are violet. (b) DNA breakage detection–fluorescence *in situ* hybridization labelling with a whole-genome probe (red fluorescence), demonstrating extensive DNA breakage in those nuclei that are intensely labelled. (c) Acridine orange stain to native DNA fluoresces green (1), whereas relaxed/denatured DNA fluoresces red (2).

92% and sensitivity reaching 42% when the threshold of 45% is used for sperm cells with abnormal nuclei [227]. TB staining has been used in several studies for evaluating sperm chromatin quality [228–231], alone and in conjunction with other tests, proving it to be an effective tool for evaluation of chromatin status.

Advantages and limitations

The TB method is simple and inexpensive and has the advantage of providing permanent preparations for use on an ordinary microscope. The stained smears can also be used for morphological assessment of the cells. Also, with the threshold for infertility diagnostics using TB staining having been established, the TB staining method is more advantageous. However, this method may have the inherent limitations of reproducibility dictated by the limited number of cells that can be reasonably scored.

CMA₃ assay

Principle

CMA₃ is a guanine–cytosine-specific fluorochrome that reveals poorly packaged chromatin in spermatozoa and is the indirect measure of protamine deficiency in sperm DNA [232]. CMA₃ is specific for GC-rich sequences and is believed to compete with protamines for binding to the minor groove of DNA. Therefore, high CMA₃ fluorescence is a strong indicator of a low protamination state in spermatozoa [206].

Technique

Sperm smears are fixed in methanol–glacial acetic acid 3:1 at 4°C for 20 minutes and are then allowed to air-dry at room temperature for 20 minutes. The slides are treated for 20 minutes with 100 µL CMA₃ solution. The CMA₃ solution consists of 0.25 mg/mL CMA₃ in McIlvain's buffer (pH 7.0) supplemented with 10 mmol/L MgCl₂. The slides are rinsed in buffer and mounted with 1:1 v/v PBS–glycerol. The slides are then kept at 4°C overnight. Fluorescence is evaluated using a fluorescent microscope. A total of 200 spermatozoa are randomly evaluated on each slide. CMA₃ staining is evaluated by distinguishing spermatozoa that stain bright yellow (CMA₃ positive) from those that stain a dull yellow (CMA₃ negative) [207, 210].

Clinical significance

As a discriminator of IVF success (>50% oocytes fertilized), CMA₃ staining has a sensitivity of 73% and a specificity of 75%.

Therefore, it can distinguish between IVF success and failure [233]. In cases of ICSI, Sakkas et al. [234] reported that the percentage of CMA₃ positivity does not indicate failure of fertilization entirely and suggested that poor chromatin packaging contributes to a failure in the decondensation process and probably reduced fertility. It appears that semen samples with high CMA₃ positivity (>30%) may have significantly lower fertilization rates if used for ICSI [235], but this observation is not seen in studies [236].

Advantages and limitations

The CMA₃ assay yields reliable results as it is strongly correlated with other assays used in the evaluation of sperm chromatin [206, 237]. CMA₃ staining results have been reported to have a strong negative correlation with sperm concentration, motility, and especially normal morphology. Men with low scores of morphologically normal spermatozoa tend to have a greater degree of protamine deficiency and DNA damage [84, 237]. The number of CMA₃-positive sperm was significantly higher in globozoopermic patients than in controls, which indicates high levels of DNA damage [232]. In addition, the sensitivity and specificity of the CMA₃ stain are comparable with those of the AAB stain (75% and 82% vs. 60% and 91%, respectively) if used to evaluate the chromatin status in infertile men [207]. However, the CMA₃ assay is limited by observer subjectivity.

DBD–FISH assay

Principle

The DBD–FISH is a technique that can detect DNA breaks in single cells, not only in the whole genome but also in specific sequences of DNA. Cells embedded within an agarose matrix on a slide are exposed to an alkaline unwinding solution, which transforms DNA strand breaks into single-stranded DNA motifs. After neutralization and protein removal, single-stranded DNA becomes accessible to hybridization with whole-genome or specific DNA probes that highlight the chromatin area to be analysed. As the number of DNA breaks increase, so does production of single-stranded DNA by the alkaline solution, resulting in an increase in fluorescence intensity and the surface area of the FISH signal. Abnormal chromatin packaging in sperm cells greatly increases the accessibility of DNA ligands and the sensitivity of DNA to denaturation by alkali, and this relates to the presence of intense labelling (red fluorescence) by DBD–FISH. Therefore,

DBD–FISH allows *in situ* detection and quantification of DNA breaks and reveals structural features in the sperm chromatin [207, 238].

Technique

Sperm cells are mixed with 1% low-melting point agarose to a final concentration of 0.7% at 37°C. A volume of 300 µL of the mixture is pipetted onto polystyrene slides and allowed to solidify at 4°C. The slides are immersed into a freshly prepared alkaline denaturation solution (0.03 mol/L NaOH, 1 mol/L NaCl) for five minutes at 22°C in the dark to generate single-stranded DNA from DNA breaks. The denaturation is then stopped, and proteins are removed by transferring the slides to a tray with neutralizing and lysing solution 1 (0.4 mol/L Tris, 0.8 mol/L dithiothreitol [DTT], 1% sodium dodecyl sulphate [SDS], and 50 mmol/L ethylenediaminetetraacetic acid [EDTA], pH 7.5) for 10 minutes at room temperature, which is followed by incubation in a neutralizing and lysing solution 2 (0.4 mol/L Tris, 2 mol/L NaCl, and 1% SDS, pH 7.5) for 20 minutes at room temperature. The slides are thoroughly washed in Tris–borate–EDTA buffer (0.09 mol/L Tris–borate and 0.002 mol/L EDTA, pH 7.5) for 15 minutes, dehydrated in sequential 70%, 90%, and 100% ethanol baths (two minutes each), and air-dried. A human whole-genome probe is hybridized overnight (4.3 ng/µL in 50% formamide/2 × standard saline citrate [SSC], 10% dextran sulphate, and 100 mmol/L calcium phosphate, pH 7.0; 1 × SSC is 0.015 mol/L sodium citrate and 0.15 mol/L sodium chloride, pH 7.0). It is then washed twice in 50% formamide/2 × SSC (pH 7.0) for five minutes and twice in 2 × SSC (pH 7.0) for three minutes at room temperature. The hybridized probe is detected with streptavidin indocarbocyanine (1:200) (Sigma Chemical Co., St Louis, MO), and cells are counterstained with 4,6-diamidino-2-phenylindole (DAPI) (1 µg/mL) and visualized using fluorescence microscopy [208].

Advantages and limitations

DBD–FISH is used to detect *in situ* DNA breaks and to reveal structural features of chromatin. Its major advantage is the possibility to simultaneously detect and discriminate single- and double-strand DNA breaks [239]. Nevertheless, it is expensive and time-consuming and involves sophisticated laboratory procedures.

In situ NT assay

Principle

The NT assay is a modified version of the TUNEL assay; it quantifies the incorporation of biotinylated dUTP at single-strand DNA breaks in a reaction that is catalysed by the template-dependent (unlike TUNEL) enzyme DNA polymerase I.

It specifically stains spermatozoa that contain appreciable and variable levels of endogenous DNA damage. The NT assay indicates anomalies that have occurred during remodelling of the nuclear DNA in spermatozoa. In doing so, it is more likely to detect sperm anomalies that are not indicated by morphology.

Technique

To perform the assay, smears containing 500 sperm each should be prepared. The fluorescent staining solution is prepared by mixing 10 µL streptavidin–fluorescein–isothiocyanate, 90 µL Tris buffer, and 900 µL double-distilled water. A total of 100 µL of this solution is added to the slides. The slides are incubated in a moist chamber at 37°C for 30 minutes. After incubation, the slides are rinsed in PBS twice, washed with distilled water, and

finally mounted with a 1:1 mixture of PBS and glycerol. The slides are examined using fluorescence microscopy. A total of 100–200 spermatozoa should be counted, and those fluorescing and hence incorporating the dye are classified as having endogenous nicks [209].

Clinical significance

Sperm nuclear integrity as assessed by the NT assay demonstrates a very clear relationship with sperm motility and morphology and, to a lesser extent, sperm concentration [182, 240, 241]. The results of the assay are supported by the strong positive correlations detected with the sensitivity of CMA₃ and TUNEL assays ($r = 0.86$, $p < 0.05$ and $r = 0.87$, $p < 0.05$, respectively) [206]. The NT assay can also indicate if there is damage arising from factors such as heat exposure [242] or the generation of ROS following exposure to leukocytes within the male reproductive tract [243].

Advantages and limitations

The advantage of the NT assay is that the reaction is based on direct labelling of the termini of DNA breaks. Thus, the lesions that are measured are identifiable at the molecular level. In addition, if flow cytometry is used to analyse the results, it may be performed on fixed cells, as the duration of cell storage in ethanol may vary [208]. However, the NT assay has a lower sensitivity than the other assays and does not correlate with fertilization in *in vivo* studies.

AO assay

Principle

AO is a dye that intercalates with DNA or RNA and fluoresces to emit different colours, making it easy to differentiate cellular organelles. The binding that occurs is the property of electrostatic interactions between acridine molecules and base pairs of nucleic acid. It measures the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* by quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA) [244]. The fluorochrome AO intercalates into double-stranded DNA as a monomer and binds to single-stranded DNA as an aggregate. The monomeric AO bound to native DNA fluoresces green, whereas the aggregated AO on relaxed or denatured DNA fluoresces red (Figure 8.4c) [245].

Technique

The AO assay can be used for either fluorescence or flow cytometry. For fluorescence microscopy, thick semen smears are fixed in Carnoy's fixative (methanol:acetic acid 1:3) for at least two hours. The slides are stained in AO for five minutes and gently rinsed with deionized water. At least 200 cells should be counted so that the estimates of the numbers of sperm with green and red fluorescence are accurate. Spermatozoa that emit green fluorescence are considered to have normal DNA content, whereas those displaying a spectrum of yellow–orange to red fluorescence are considered to have damaged DNA. The DNA fragmentation index (DFI) can be calculated by the ratio of (yellow to red)/(green + yellow to red) fluorescence [244].

For flow cytometry, aliquots of semen (about 25–100 µL, containing one million spermatozoa) are suspended in 1 mL of ice-cold PBS (pH 7.4) and centrifuged at 600 g for five minutes. The pellet is resuspended in ice-cold TNE (0.01 mol/L Tris-HCl, 0.15 mol/L NaCl, and 1 mmol/L EDTA, pH 7.4) and again centrifuged at 600 g for five minutes. The pellet is then resuspended in 200 µL of ice-cold TNE with 10% glycerol and immediately fixed

in 70% ethanol for 30 minutes. The fixed samples are treated for 30 seconds with 400 µL of a solution of 0.1% Triton X-100, 0.15 mol/L NaCl, and 0.08 N HCl (pH 1.2). After 30 seconds, 1.2 mL of staining buffer (6 µg/mL AO, 37 mmol/L citric acid, 126 mmol/L Na₂HPO₄, 1 mmol/L disodium EDTA, and 0.15 mol/L NaCl, pH 6.0) is added to the test tube and analysed by flow cytometry. After excitation by a 488-nm wavelength light source, AO bound to double-stranded DNA fluoresces green (515–530 nm) and AO bound to single-stranded DNA fluoresces red (630 nm or greater). A minimum of 5000 cells are analysed by fluorescent-activated cell sorting [209].

Clinical significance

The AO technique has shown significantly higher DNA damage in infertile men with and without varicocele as compared to controls [230]. Further, a decrease in AO-positive spermatozoa has also been documented after varicocelectomy, which shows its clinical utility in the evaluation of DNA integrity [148]. AO-positive cells are likely to have more structural abnormalities than AO-negative cells [246]. A negative correlation has been reported between AO staining results and conventional sperm parameters [247]. The “cut-off” value set to differentiate between fertile and infertile men varies between 20% and 50% [17, 210]. Studies show that single-stranded DNA that is detected by a low incidence (<50%) of green AO fluorescence negatively affects the fertilization process in a classical IVF program, resulting in lower fertilization and pregnancy rates and a lower proportion of grade A embryos [173, 248, 249]. However, no correlation was found with the pregnancy rate and live births achieved by ICSI except in patients having 0% of spermatozoa with single-stranded DNA, in whom the pregnancy rate was significantly higher [173, 248, 249].

Advantages and limitations

The AO assay is a biologically stable measure of sperm DNA quality. The intra-assay variability is less than 5%, rendering the technique highly reproducible [250]. A strong positive correlation exists between the AO assay and other techniques used to evaluate single-stranded DNA (e.g. the TUNEL assay [see the “TUNEL assay” section]) [250]. Limitations include inter-observer variability in case of fluorescence microscopic analysis and expensive instrumentation for flow cytometric analysis.

SCD test (Halosperm® assay)

Principle

The SCD test produces sperm nucleoids consisting of a central or core and peripheral halo caused by release of DNA loops, signifying the absence of DNA fragmentation. When sperm are treated with an acid solution prior to lysis buffer, a complete absence or a minimal halo is produced in spermatozoa with fragmented DNA. A distinct halo is seen in spermatozoa with intact DNA integrity [211]. When spermatozoa with non-fragmented DNA are immersed in an agarose matrix and directly exposed to lysing solutions, the resulting deproteinized nuclei (nucleoids) show extended halos of DNA dispersion, which can be observed either by bright field microscopy or fluorescence microscopy. The presence of DNA breaks promotes the expansion of the halo of the nucleoid [119, 251–256] (Figures 8.5 and 8.6).

Technique

Aliquots of sperm at a concentration of 5–10 million/mL are prepared by diluting in PBS. The samples are mixed with 1% low-melting point aqueous agarose (to obtain a 0.7% final agarose concentration) at 37°C. Aliquots of 50 µL of the mixture

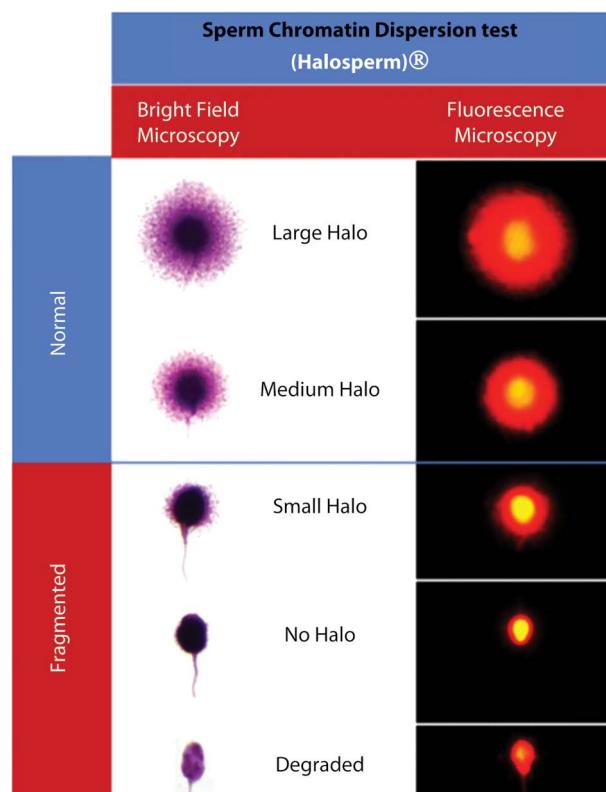


FIGURE 8.5 Classification of human SDF with respect to halo size and visualization under bright field or fluorescence microscopy using Halosperm®. Abbreviation: SDF, sperm DNA fragmentation.

are pipetted onto a glass slide precoated with 0.65% standard agarose dried at 80°C, covered with a coverslip, and left to solidify at 4°C for four minutes. The coverslips are then carefully removed, and the slides are immediately immersed horizontally in a tray of freshly prepared acid denaturation solution

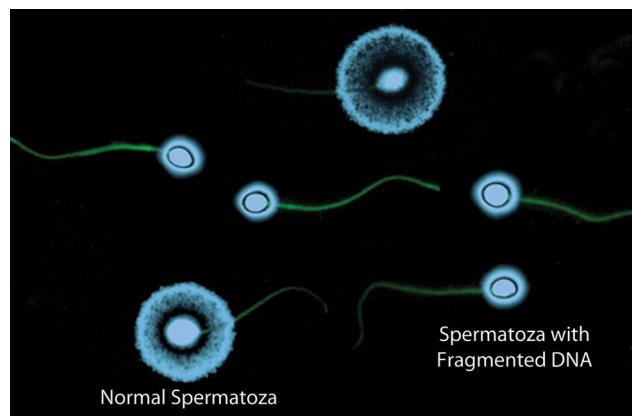


FIGURE 8.6 Visualization of SDF using Halosperm. Sperm was simultaneously stained for protein visualization (green) and DNA (blue). This image was electronically filtered for halo enhancement and discrimination between sperm containing fragmented DNA (no halo of dispersed chromatin) and non-fragmented DNA (large halo of dispersed chromatin). Abbreviation: SDF, sperm DNA fragmentation.

(0.08 N HCl) for seven minutes at 22°C in the dark, which generates restricted single-stranded DNA motifs from DNA breaks. Denaturation is then stopped, and the proteins are removed by transferring the slides to a tray with neutralizing and lysing solution 1 (0.4 mol/L Tris, 0.8 mol/L DTT, 1% SDS, and 50 mmol/L EDTA, pH 7.5) for 10 minutes at room temperature. The slides are then incubated in neutralizing and lysing solution 2 (0.4 mol/L Tris, 2 mol/L NaCl, and 1% SDS, pH 7.5) for five minutes at room temperature. The slides are thoroughly washed in Tris–borate EDTA buffer (0.09 mol/L Tris–borate and 0.002 mol/L EDTA, pH 7.5) for two minutes; dehydrated sequentially in 70%, 90%, and 100% ethanol baths (two minutes each); and air-dried. Cells are stained with DAPI (2 µg/mL) for fluorescence microscopy (Figures 8.5 and 8.6).

Clinical significance

Reports suggest that SDF as reported by the SCD test is negatively correlated with fertilization rates and embryo quality in IVF/ICSI, but not with clinical pregnancy rates or births [252–257]. A meta-analysis examining the outcomes of medically assisted reproduction failed to show any predictive value of SCD between IVF and ICSI [258]. Simon et al. [23] demonstrated a negative effect of sperm DNA damage on clinical pregnancy rate following IVF and ICSI, whereas Deng et al. [33], demonstrated the negative effect of lower pregnancy rate only in IVF. In another study by Ribas-Maynou [259], DNA damage by SCD adversely affected only pregnancy and live birth rate but not implantation rate, fertilization rate, embryo quality, or blastocyst formation in IVF; but none of these parameters were affected in ICSI.

Advantages and limitations

The SCD test is simple, fast, and reproducible, with comparable results to those of the SCSA [251, 254] and TUNEL assay [260]. The currently available protocol is suitable for bright field microscopy as it significantly reduces equipment cost. The test is successfully used in clinical studies to detect sperm DNA damage [261] and can be simultaneously combined with the FISH (SCD–FISH) assay for detection of aneuploidy in sperm cells [262]. This is the only test allowing assessment of SDF and chromosomal aneuploidy by FISH in the same cell. Oxidative DNA damage also can be simultaneously determined in the same sperm cell by combining the SCD test and incubation with an 8-oxoguanine DNA probe [263]. A commercially available Halosperm® kit has been recently developed [121, 264].

Comet assay

Principle

The comet assay (single-cell gel electrophoresis) was first introduced by Ostling and Johanson in 1984 [265] and is based on the principle of permeabilization and electrophoretic migration of cleaved fragments of DNA [266]. In the beginning, neutral electrophoresis buffer conditions were used to show that the migration of double-stranded DNA loops from a damaged cell in the form of a tail unwinding from the relaxed supercoiled nucleus was proportional to the extent of damage inflicted on the cell. This finding took on the appearance of a comet with a tail when viewed using a fluorescence microscope and DNA stains. Singh et al. modified the comet assay in 1988 [212] by using alkaline electrophoresis buffers to expose alkali-labile sites on the DNA; this modification increased the sensitivity of the assay to detect both single- and double-stranded DNA breaks [212]. The routine comet assay lacks the ability to differentiate between single- and

double-stranded DNA breaks in the same sperm cell, but a modified two-tailed comet assay can simultaneously evaluate single- and double-stranded DNA breaks [267]. The chromosome comet assay is a new application that detects DNA damage by generating comets in sub-nuclear units, such as the chromosome, based on the chromosome isolation protocols currently used for whole-chromosome mounting in electron microscopy. It has not been used with sperm cells thus far [268].

In the comet assay, DNA damage is quantified by measuring the displacement between the genetic material of the nucleus “comet head” and the resulting tail. The tail lengths are used as an index for the damage. Also, the tail moment—the product of the tail length and intensity (fraction of total DNA in the tails)—has been used as a measuring parameter. The tail moment can be more precisely defined as being equivalent to the torsional moment of the tail [269].

Technique

Sperm cells are cast into miniature agarose gels on microscopic slides and lysed *in situ* to remove DNA-associated proteins in order to allow the compacted sperm DNA to relax. The lysis buffer (Tris 10 mmol/L, 0.5 mol/L EDTA, and 2.5 mol/L NaCl, pH 10) contains 1% Triton X-100, 40 mmol/L DTT, and 100 µg/mL proteinase K. The slide immersion time in alkaline lysis solution ranges between 1 and 20 minutes and does not affect assay results [270]. Micro-gels are then electrophoresed (20 minutes at 25 V/0.01 A) in neutral buffer (Tris 10 mmol/L containing 0.08 mol/L boric acid and 0.5 mol/L EDTA, pH 8.2), during which time the damaged DNA migrates from the nucleus towards the anode. The DNA is visualized by staining the slides with the fluorescent DNA binding dye SYBR Green I. Comet measurements are performed manually or by computerized image analysis using fluorescence microscopy (Figure 8.7) [211].

In the two-tailed comet technique, sperm cells are diluted in PBS to a concentration of 10×10^6 spermatozoa/mL. A 25-µL cell suspension is mixed with 50 µL of 1% low-melting point agarose in distilled water at 37°C. A total of 15 µL of the mixture is placed on the slide, covered with a coverslip, and transferred to an ice-cold plate. As soon as the gel solidifies, the coverslips are removed and the slides are rinsed in two lysing solutions: lysing solution 1 (0.4 mol/L Tris–HCl, 0.8 mol/L DTT, and 1% SDS, pH 7.5) for 30 minutes, followed by lysing solution 2 (0.4 mol/L Tris–HCl, 2 mol/L NaCl, 1% SDS, and 0.05 mol/L EDTA, pH 7.5) for 30 minutes. Then, the slides are rinsed in TBE buffer (0.09 mol/L Tris–borate and 0.002 mol/L EDTA, pH 7.5) for 10 minutes, transferred to an electrophoresis tank, and immersed in fresh TBE electrophoresis buffer. Electrophoresis is performed at 20 V (1 V/cm) and 12 mA for 12.5 minutes. After washing in 0.9% NaCl, nucleoids are unwound in an alkaline solution (0.03 mol/L NaOH and 1 mol/L NaCl) for 2.5 minutes, transferred to an electrophoresis chamber, and oriented at 90° to the first electrophoresis.

The second electrophoresis is performed at 20 V (1 V/cm), and 12 mA for four minutes in 0.03 mol/L NaOH. Then, the slides are rinsed in a neutralization buffer (0.4 mol/L Tris–HCl, pH 7.5) for five minutes, briefly washed in TBE buffer, dehydrated in increasing concentrations of ethanol, and air-dried. DNA is stained with SYBR Green I at a 1:3000 dilution in Vectashield® (Vector Laboratories, Burlingame, CA). Samples are assessed by visual scoring or digitalization and image processing. The frequency of sperm cells with fragmented DNA is established by measuring at least 500 sperm cells per slide. Cells are classified as undamaged

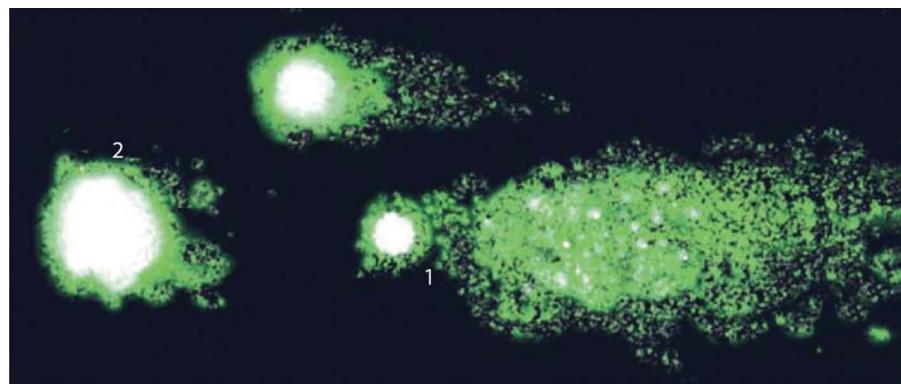


FIGURE 8.7 Comet image showing damaged DNA (1) and undamaged DNA (2).

or damaged based on the length of the tail, which contains DNA fragment single-stand breaks (up/down migration), DSBs (right/left migration), or both [267].

Clinical significance

The assay has been successfully used to evaluate DNA damage after cryopreservation [271]. Using the alkaline and neutral comet assay also showed that normozoospermic men with varicoceles have high single- and double-strand DNA damage [148]. ROS are directly responsible for damage to the DNA single and double strands. It may also predict embryo development after IVF and ICSI, especially in couples with unexplained infertility [272], and some clinical thresholds were set for infertility diagnostics and IVF outcome prediction [273–274], although some studies failed to demonstrate such an association [275]. DNA damage by alkaline comet assay was also shown to negatively affect pregnancy rate and/or live-birth rate in IVF but not in ICSI [20, 23, 33, 259, 276, 277]. A modified version of the comet assay protocol is capable of detecting different mutagen impacts on sperm DNA integrity [278]. Alkaline comet assay has been demonstrated as a robust biomarker for sporadic and recurrent miscarriages after spontaneous or assisted conception [22].

Advantages and limitations

The comet assay is a well-standardized, simple, versatile, sensitive, and rapid assay that correlates significantly with the TUNEL assay and SCSA [279]. It can assess DNA damage qualitatively as well as quantitatively with low intra-assay variation. Two-tailed comet assay can discriminate between single- and double-stranded DNA breaks; for example, the resistance of sperm DNA to oxidative damage can be specifically assessed [280]. Because it is based on fluorescence microscopy, the assay requires an experienced observer to analyse the slides and interpret the results.

TUNEL assay

Principle

This single-step staining method labels DNA breaks with fluorescein isothiocyanate (FITC)-dUTP followed by flow cytometric analysis. TUNEL utilizes a template-independent DNA polymerase called TdT, which non-preferentially adds deoxyribonucleotides to 3'-hydroxyl (OH) single- and double-stranded DNA. dUTP is the substrate that is added by the TdT enzyme to the free 3'-OH break-ends of DNA (Figure 8.8).



FIGURE 8.8 BD Accuri C6 flow cytometer.

Technique

Strand breaks can be quantified with conventional or the newly introduced benchtop flow cytometry or fluorescence microscopy in which DNA-damaged sperm fluoresce intensely [164, 281]. To assess the DNA fragmentation by TUNEL, an APO-DIRECT™ Kit (BD Pharmingen, CA, USA) is used. It contains the reaction buffer, TdT, FITC-dUTP, and propidium iodide/RNase stain. The assay kit also contains negative and positive controls, which are not sperm cells. About 5×10^6 sperm cells are fixed with 3.7% paraformaldehyde for a minimum of 30 minutes at 4°C. The sample is centrifuged at 300 g for seven minutes. Paraformaldehyde is removed by centrifuging the samples at 300 g for seven minutes. Supernatants are discarded and the pellets resuspended with 1 mL of ice-cold ethanol (70% v/v). The tubes are kept at -20°C for at least 30 minutes. To create negative sperm controls, the enzyme terminal transferase is omitted from the reaction mixture. To create positive sperm controls, the samples are pre-treated with 0.1 IU DNase I for 30 minutes at room temperature. A total of 50 µL of the stain is added and incubated for one hour. Following two washes with 1 mL of the "rinse buffer," propidium iodide/RNase stain is added and incubated for 30 minutes. For flow cytometry, two laser detectors are used: FL1 (488) with a standard 533/30 band-pass (BP) that detects green fluorescence, and FL2 with a standard 585/40 BP that detects red or propidium iodide fluorescence. The tubes are analysed for DNA fragmentation using the BD Accuri™ C6 flow cytometer (BD cytometers, USA) (Figure 8.9). A quality control assay is also run using the eight-peak beads as per the manufacturer's instructions. Although less accurate, the samples can also be assessed by scoring about 500 sperm cells under fluorescence microscopy [164, 281].

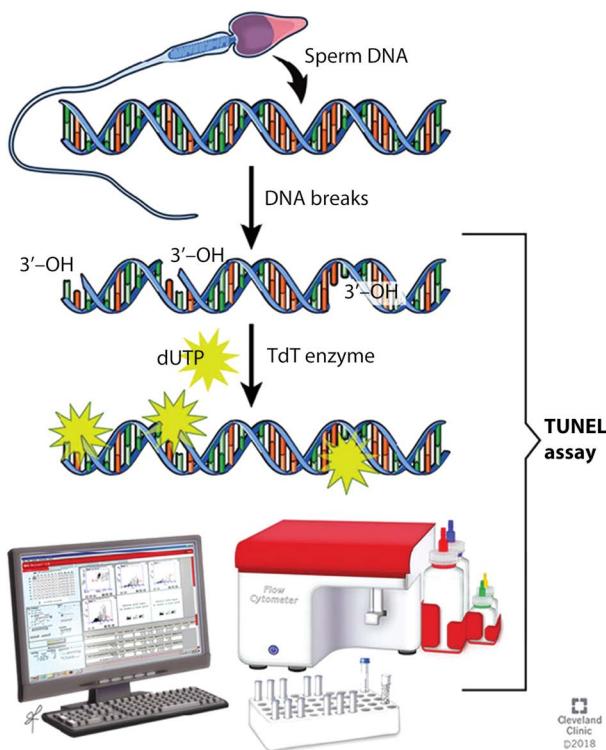


FIGURE 8.9 Schematic of DNA staining using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay.

The standard TUNEL assay can be improved to become more sensitive to DNA fragmentation by incubating sperm cells in 2 mM DTT solution for 45 minutes prior to fixation with formaldehyde. This modified version of the TUNEL assay was shown to significantly enhance its sensitivity. Mitchell et al. modified the TUNEL methodology by incubating spermatozoa for 30 minutes at 37°C with LIVE /DEAD™ Fixable Dead Cell Stain (far red) (Molecular Probes, Eugene, OR). The cells were then washed three times with Biggers–Whitten–Whittingham medium (BWW) before incubation with DTT; this allowed both DNA integrity and vitality to be simultaneously assessed [282].

Clinical significance

The TUNEL assay has been widely used in male infertility research related to SDF. A negative correlation was found between the percentage of DNA-fragmented sperm and motility, morphology, and concentration in the ejaculate. It also appears to be potentially useful as a predictor for IUI pregnancy rates, IVF embryo cleavage rates, and ICSI fertilization rates. In addition, it provides an explanation for recurrent pregnancy loss [18, 279, 283]. A cut-off value of 19.2% has been shown significant differentiation between fertile and infertile men with a sensitivity of 64.9% and a specificity of 100% [174, 279]. This is higher than that demonstrated for IUI procedures (12%) [284]. A very high specificity (91.6%) and positive predictive value (PPV) (1.40%) at a cut-off point of 16.8% [163]. The high specificity of the TUNEL assay is helpful in correctly identifying infertile patients who do not have SDF as a contributory factor [214]. Due to its high positive predictive value, the assay is able to confirm that a man who tests positive is likely to be infertile due to elevated SDF (Figure 8.10a and b) [285]. The calculated cut-off would be ideal as any value above this threshold will be strongly associated with infertility.

Advantages and limitations

The TUNEL assay is relatively expensive and time and labour consuming. Also, a number of factors can significantly affect assay results, including the type and concentration of fixative, fixed sample storage time, the fluorochrome used to label DNA breaks, and the method used to analyse flow cytometric data [286]. The flow cytometric method of assessment is generally more accurate and reliable than fluorescence microscopy, but it is also more sophisticated and expensive and it presents limitations in the accuracy and reproducibility of the measures of SDF [163, 287]. Fairly good-quality control parameters with minimal inter- and intra-observer variation (<8%) have been reported [163]. Similarly, the two flow cytometers showed very high precision (98%) and accuracy (>99%) along with interobserver agreement, establishing the robustness of both instruments [216].

Sperm chromatin structure assay

Principle

The SCSA measures *in situ* DNA susceptibility to the acid-induced conformational helix-coil transition by AO fluorescence staining. Acridine orange easily penetrates the dense chromatin and intercalates with the double-stranded DNA. This fluoresces green under blue laser light at 488 nm. The extent of conformational transition *in situ* following acid or heat treatment is determined by measuring the metachromatic shift of AO fluorescence from green (native DNA) at 515–530 nm under blue laser light to red (denatured or relaxed DNA) under red laser >630 nm. This protocol has been divided into SCSA_{acid} and SCSA_{heat} in order to distinguish the physical means of inducing conformational

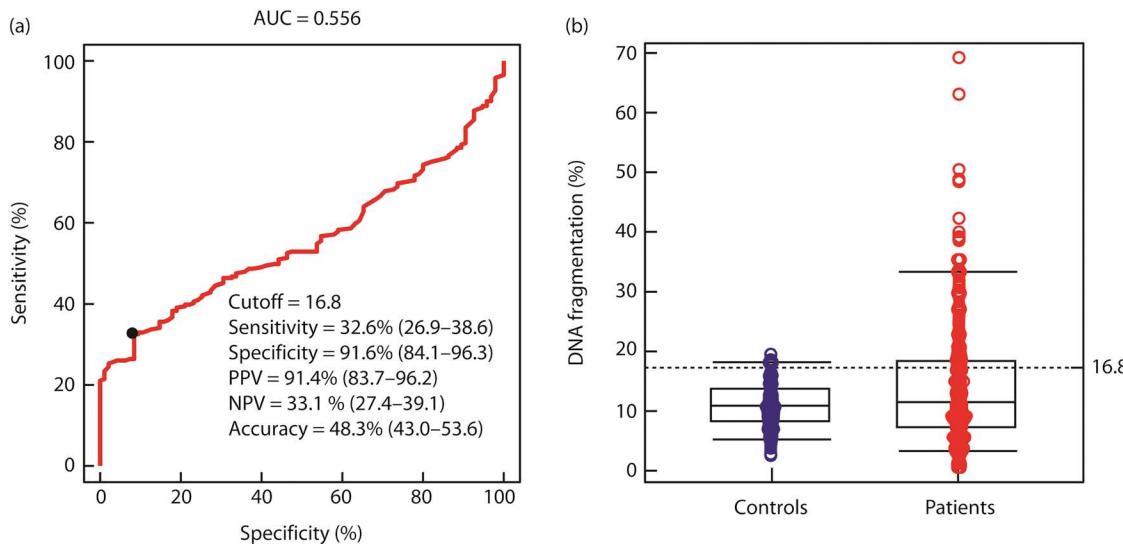


FIGURE 8.10 (a) Receiver operator characteristic curve showing terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) cut-off and the area under the curve (AUC). Values within the parentheses represent 95% confidence intervals. (b) Distribution of TUNEL values between controls and infertile men. Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

transition. The two methods give essentially the same results, but the SCSA_{acid} method is easier to use.

Technique

To perform SCSA, an aliquot of unprocessed semen (about 13–70 µL) is diluted to a concentration of 1–2 × 10⁶ sperm/mL with TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, and 1 mM EDTA, pH 7.4). This cell suspension is treated with an acid detergent solution (pH 1.2) containing 0.1% Triton X-100, 0.15 mol/L NaCl, and 0.08 N HCl for 30 seconds and then stained with 6 mg/L purified AO in a phosphate–citrate buffer (pH 6.0). The stained sample is placed into the flow cytometer sample chamber [17]. The assay measures 5000 sperm by flow cytometry and DNA damage is indicated by the % DNA Fragmentation Index (DFI), which is a ratio of red fluorescence divided by total (red + green) fluorescence [17].

Clinical significance

Because the SCSA results are more constant over prolonged periods of time than routine World Health Organization (WHO) semen parameters, it may be used effectively in epidemiological studies of male infertility [288]. No significant male age-related increase in DFI has been demonstrated [289]. Currently, the SCSA is the only assay that has clearly established clinical thresholds for utility in the human infertility clinic [290]. In clinical applications, the SCSA parameters not only distinguish fertile and infertile men but also are able to classify men according to the level of *in vivo* fertility as high fertility (pregnancy initiated in less than three months), moderate fertility (pregnancy initiated within 4–12 months), and no proven fertility (no pregnancy by 12 months). In addition, a DFI threshold was established that identifies samples that are compatible with *in vivo* pregnancy (<30%) [12, 290–293].

The SCSA has been considered a gold standard as a robust assay for measuring DNA damage by flow cytometry and can predict various ART outcomes. However, the ability to predict ART outcomes, including fertilization and implantation rates, is true only for neat semen [9, 12, 187, 294]. An increased abortion rate in the

high-DFI (>27%) group has been reported [275]. It has also been suggested that DFI can be used as an independent predictor of fertility in couples undergoing IUI [9], but the association between SCSA results and IVF and ICSI outcomes is not strong enough [295]. It has also been proposed that all infertile men should be tested with the SCSA in addition to standard semen analysis [296] and, if DFI is higher than 30%, ICSI should be recommended [12]. The SCSA also measures sperm with high DNA stainability, which is related to the nuclear histones retained in immature sperm. Sperm with high DNA stainability is reported to be predictive of pregnancy failure [297]. The current clinical threshold has changed from >30% to 25% DFI. DNA fragmentation above 25% categorizes a patient into the following statistical probabilities: (i) longer time to natural pregnancy; (ii) low odds of IUI pregnancy; (iii) more miscarriages; or (iv) no pregnancy [297]. The SCSA is considered to be a precise and repeatable DNA fragmentation test that can reliably identify a man who is at risk for infertility.

Advantages and limitations

The SCSA accurately estimates the percentage of DNA-damaged sperm and has a cut-off point (30% DFI) to differentiate between fertile and infertile samples. This has been recently revised to 25% [9, 290, 297]. However, it requires the presence of expensive instrumentation (flow cytometer) and highly skilled technicians. The SCSA DFI is significantly associated with TUNEL assay results when Spearman's rank correlation is used. However, regression and concordance correlation results showed that these methods are not comparable. The SCSA measures DNA damage in terms of susceptibility to DNA denaturation, whereas TUNEL measures "real" DNA damage [298].

Measurement of 8-OHdG

Principle

This assay measures levels of 8-OHdG, which is a by-product of oxidative DNA damage, in spermatozoa. It is the most commonly studied biomarker for oxidative DNA damage. Among various oxidative DNA adducts, 8-OHdG has been selected as a

representative of oxidative DNA damage owing to its high specificity, potent mutagenicity, and relative abundance in DNA [299].

Technique

Step I

DNA extraction is performed with chloroform–isoamyl alcohol (12:1 v/v) after the sperm cells are washed with sperm wash buffer (10 mmol/L Tris-HCl, 10 mmol/L EDTA, and 1 mol/L NaCl, pH 7.0) and lysed at 55°C for one hour with 0.9% SDS, 0.5 mg/mL proteinase K, and 0.04 mol/L DTT. After ribonuclease A treatment to remove RNA residue, the extracted DNA is dissolved in 10 mmol/L Tris-HCl (pH 7.0) for DNA digestion.

Step II

Enzymatic DNA digestion is performed with three enzymes: DNase I, nuclease P1, and alkaline phosphatase. The final solution is dried under reduced temperature and pressure and is re-dissolved in distilled and deionized water for HPLC.

Step III

The third step is HPLC analysis. The HPLC system used for 8-OHdG measurements consists of a pump, a Partisphere® 5 C18 column (Hichrom Limited, UK), an electrochemical detector, a UV detector, an autosampler, and an integrator. The mobile phase consists of 20 mmol/L NH₄H₂PO₄, 1 mmol/L EDTA, and 4% methanol (pH 4.7). The calibration curves for 8-OHdG are established with standard 8-OHdG, and the results are expressed as 8-OHdG/10⁴ dG [218].

Clinical significance

The assay provides the most direct evidence suggesting that oxidative sperm DNA damage is involved in male infertility based on the finding that 8-OHdG levels in sperm are significantly higher in infertile patients than in fertile controls and are inversely correlated with sperm concentration [300]. 8-OHdG formation and DNA fragmentation as assessed by TUNEL are highly correlated with each other [301]. 8-OHdG levels also are highly correlated with the disruption of chromatin remodelling [122]. Levels of 8-OHdG in sperm DNA have been reported to be increased in smokers, and they are inversely correlated with the intake and seminal plasma concentration of vitamin C—the most important antioxidant in sperm. Infertile patients with varicocele have increased 8-OHdG expression in the testis, which is associated with deficient spermatogenesis [302]. If not repaired, 8-OHdG modifications in DNA are mutagenic and may cause embryo loss, fetal malformations, or childhood cancer. Moreover, these modifications could be a marker of OS in sperm, which may have negative effects on sperm function [303, 304].

Advantages and limitations

Although 8-OHdG is a potential marker for oxidative DNA damage, artificial oxidation of dG can occur during analysis, which can lead to inaccurate results. A fixed number of sperm cells should be analysed as a precaution. However, the DNA yield cannot be excluded as a potential confounder.

Clinical utility of sperm DNA fragmentation

In the past decade, there has been mounting evidence that supports the clinical utility of DNA tests in male fertility evaluation [30, 139, 148, 180, 297]. Due to distinct assay characteristics, the results obtained from one method do not necessarily match those provided by other tests. There seems to be a fairly good correlation among the three widely used tests, namely SCSA, TUNEL,

and SCD, although the evidence is not unequivocal. Emerging evidence indicates that SDF has a clear influence on the reproductive outcomes, both naturally and via assisted reproductive techniques. A recently published practice recommendation by Agarwal et al. represents the first attempt to propose specific clinical indications for SDF testing. These indications were clinical varicocele, unexplained infertility/IUI failure or recurrent pregnancy loss, IVF/ICSI failure, and lastly borderline abnormal or normal semen parameters with risk factors [180]. This consensus statement was later endorsed by the Society for Translational Medicine and Clinical Practice Guidelines (CPG) for SDF testing in male fertility in 2017 [162]. Utilizing a Strengths-Weakness-Opportunity-Threat (SWOT) analysis to understand the perceived advantages and drawbacks of SFDF as a specialized function test in clinical practice [34], the analysis revealed that CPG provides reasonable evidence-based proposal for integration of SDF testing in routine daily practice and provides opportunity to further improve SDF testing.

The WHO manual provides a detailed description of different tests used in the evaluation of SDF [41] but does not provide a clinical description of the possible indications for SDF testing. On the other hand, the indications governing the clinical utility of SDF testing have been clearly defined in the recent EAU guidelines [36]. It clearly establishes SDF testing in (i) nonazoospermic men with unexplained infertility (strong recommendation) and (ii) couples with RPL (from natural conception and ARTs) (strong recommendation). In addition, two research groups have reported and published the guidelines on the indications of SDF testing [31, 32, 35]. These two reports also recommend indications for SDF testing in cases of unexplained, male infertility (Grade B and C), varicocele (Grade C), RPL (Grade B-C), RPL (Grade B-C), and ART before and after failure [35]. High SDF is also linked with recurrent pregnancy loss, elevated levels of DNA damage, and miscarriages [22]. In men with high SDF, the use of testicular sperm has also been recommended in cases of oligozoospermia or recurrent pregnancy loss [305, 306].

Men can also benefit from lifestyle modifications and SDF testing [38]. The results of SDF testing may change the management decision by selecting the most appropriate ART with the highest success rate for infertile couples.

SDF testing should be considered as one of the tests in a panel of male fertility assessment rather than a stand-alone test. A combination of a selected panel of tests, when appropriately applied, could offer additional complementary information for making a clinical diagnosis while considering various male and female factors in a clinical scenario. SDF assays have been adopted by many andrology laboratories worldwide, and gaps in our knowledge have been identified and recommendations made to further improve the clinical utility of SDF in clinical practice [143, 162].

Strategies to reduce sperm DNA damage

In view of the impact SDF has on reproductive outcomes, it is important to develop and implement appropriate treatment methods, preventive measures, and strategies to minimize DNA damage in the spermatozoa used in assisted reproduction [307]. Some of the strategies include:

1. *Appropriate sperm preparation methods:* Most of the commonly used methods such as density gradient centrifugation, swim up, and glass wool filtration yield sperm with better DNA integrity than native semen [14]. Sperm

- preparation should be aimed at minimizing damage to the spermatozoa and can be accomplished by exercising some simple precautions, such as (i) slow dilution of the samples, especially when using cryopreserved spermatozoa; (ii) gradual changes in temperature and tests performed at 37°C; (iii) minimal use of centrifugation and, when necessary, it being performed at the lowest possible speed; and (iv) controlled exposure to potentially toxic materials. Plastic ware, glassware, media, and gloves should be checked for potential toxicity as the spermatozoa may be immobilized when in contact with any potential toxic substances in these materials. In patients who are unable to produce a semen sample by masturbation, use of non-toxic condoms is important, and when necessary, a second sample should be collected a few hours after the first.
2. *Electrophoretic separation of sperm:* This is based on the principle that high-quality spermatozoa tend to be viable and morphologically normal and have a low degree of DNA fragmentation as measured by TUNEL assay [308].
 3. *Antioxidant treatments:* One of the causes of sperm DNA damage is OS. Studies have investigated the ability of antioxidant treatments to manage male subfertility, both *in vivo* and *in vitro*. It is generally accepted that antioxidants may be beneficial for reducing sperm DNA damage, but their exact mechanism of action is still not established, and some studies have reported adverse effects such as increased sperm chromatin decondensation [309, 310]. Significant improvement in clinical pregnancy and implantation rates have been shown in patients with high sperm DNA damage as assessed by TUNEL assay when treated with antioxidants before assisted reproduction [311, 312]. Therefore, in patients in whom OS is the cause of sperm DNA damage, adequate oral antioxidant supplementation appears to be a simple strategy to enhance sperm genome integrity and reproductive outcomes. Standard and reliable oral antioxidant treatment protocols and alternative treatment strategies for non-responders are needed [312].
 4. *Magnetic cell separation:* Magnetic cell separation is a useful technique to separate apoptotic and non-apoptotic spermatozoa [313].
 5. *High-magnification ICSI for patients with SDF:* It is possible to observe spermatozoa with apparently normal morphology and intranuclear vacuoles that appear to be associated with chromatin packaging by using inverted microscopes with Nomarski differential interference contrast optics combined with digitally enhanced secondary magnification [314, 315].
 6. *PICSI:* Hyaluronan-bound sperm are thought to reflect higher maturity, acrosome activity, along with lower aneuploidies and SDF [316, 317]. PICSI is highly specific and thought to improve successful pre-implantation embryogenesis [318].
 7. *Microfluidics:* By controlling fluid dynamics, it is possible to mimic the physiological conditions of pH and temperature of the female genital tract [319]. This allows the selection of spermatozoa with high motility, while debris and dead spermatozoa can be easily separated [320]. This results in significant improvement in sperm motility, morphology, and increased pregnancy rates in couples undergoing ICSI [321] and Chapter 28.
 8. *Use of testicular sperm:* Spermatozoa retrieved from the testis in men with high SDF in neat semen tend to have

better DNA quality compared to ejaculated sperm. These men may benefit from testis-ICSI if male partners have confirmed high SDF in the ejaculate [4, 143, 305, 322].

9. *Lifestyle modifications:* Infertile men with modifiable lifestyle risk factors such as smoking and obesity and high SDF can benefit from lifestyle modifications [38, 323].

Conclusion

The importance of assessing sperm chromatin integrity is well established, and the results provide useful information in cases of male idiopathic infertility and in couples pursuing assisted reproduction. Pathologically increased SDF is one paternal-derived cause of repeated assisted reproduction failures in the ICSI era. Several studies have demonstrated that sperm DNA integrity correlates with pregnancy outcome in IVF. Therefore, SDF should be included in the evaluation of the infertile male especially in infertile men as well as normozoospermic men with clinical varicoceles, and men with idiopathic infertility. Assessment of sperm DNA damage appears to be a potential tool for evaluating semen samples prior to their use in assisted reproduction. It allows for the selection of spermatozoa with intact DNA or with the least amount of DNA damage for use in assisted conception. It provides better diagnostic and prognostic capabilities than standard sperm parameters for assessing male fertility potential.

There are multiple assays that can be used to evaluate sperm chromatin. Most of these assays have advantages along with limitations. Choosing the right assay depends on many factors, such as the expense, the available laboratory facilities, and the presence of experienced technicians. The establishment of a cut-off point between normal levels in the average fertile population and minimal levels of sperm DNA integrity required for achieving pregnancy is still debated and a single cut-off may not be ideal as different tests measure different aspects of DNA fragmentation. Further research is needed on the clinical SDF thresholds to be used with each SDF test on IUI, IVF, and ICSI, using different endpoints (e.g. live birth, miscarriage). Given the importance of sperm DNA integrity, it is important to determine the real cause of DNA damage and provide proper therapeutic treatments. Methods for selecting sperm with undamaged DNA should be designed, especially in cases where ICSI is strongly recommended. With further refinements in SDF testing and emerging supporting evidence, SDF testing will help improve ART success and the health of both fathers to be and the resulting offspring.

References

1. Agarwal A, Sharma RK, Nallella KP, et al. Reactive oxygen species as an independent marker of male factor infertility. *Fertil Steril.* 2006;86:878–85.
2. Sharma R, Martinez MP, Agarwal A. Sperm chromatin integrity tests and indications. In: Male Infertility. Parekattil SJ, et al. (eds.). CH 8, pp 99–121, Springer Nature Switzerland AG, 2020.
3. Kandil K, Farkouh A, Saleh R, Boitrelle F, et al. Sperm DNA fragmentation and male infertility: A comprehensive review for the clinicians. In: Handbook of Current and Novel Protocols for the Treatment of Infertility. Dahan M, Fatemi H, Polyzos N, Garcia Velasco J, (eds.). 2023 (in press). Elsevier Science & Technology.
4. Esteves SC, Roque M, Bedoschi G, et al. Intracytoplasmic sperm injection for male infertility and consequences for offspring. *Nat Rev Urol.* 2018;15:535–62.
5. Rumbold AR, Sevyan A, Oswald TK, et al. Impact of male factor infertility on offspring health and development. *Fertil Steril.* 2019;111:1047–53.

6. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update*. 2003;9:331–45.
7. Agarwal A, Allamaneni SS. Sperm DNA damage assessment: A test whose time has come. *Fertil Steril*. 2005;84:850–3.
8. Erenpreiss J, Spano M, Erenpreisa J, et al. Sperm chromatin structure and male fertility: Biological and clinical aspects. *Asian J Androl*. 2006;8:11–29.
9. Evenson D, Wixon R. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. *Reprod Biomed Online*. 2006;12:466–72.
10. Borini A, Tarozzi N, Bizzaro D, et al. Sperm DNA fragmentation: Paternal effect on early post-implantation embryo development in ART. *Hum Reprod*. 2006;21:2876–81.
11. Aitken RJ, De Iulisi GN. Origins and consequences of DNA damage in male germ cells. *Reprod Biomed Online*. 2007;14: 727–33.
12. Bungum M, Humaidan P, Axmon A, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod*. 2007;22:174–9.
13. Ozmen B, Koutlaki N, Youssry M, et al. DNA damage of human spermatozoa in assisted reproduction: Origins, diagnosis, impacts and safety. *Reprod Biomed Online*. 2007;14:384–95.
14. Tarozzi N, Bizzaro D, Flamigni C, et al. Clinical relevance of sperm DNA damage in assisted reproduction. *Reprod Biomed Online*. 2007;14:746–57.
15. Lopes S, Jurisicova A, Sun JG, et al. Reactive oxygen species: Potential cause for DNA fragmentation in human spermatozoa. *Hum Reprod*. 1998;13:896–900.
16. Sakkas D, Tomlinson M. Assessment of sperm competence. *Semin Reprod Med*. 2000;18:133–9.
17. Evenson DP, Jost LK, Marshall D, et al. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod*. 1999;14:1039–49.
18. Aitken RJ. The Amoroso Lecture. The human spermatozoon—A cell in crisis? *J Reprod Fertil*. 1999;115:1–7.
19. Robinson L, Gallos ID, Conner SJ, et al. The effect of sperm DNA fragmentation on miscarriage rates: A systematic review and meta-analysis. *Hum Reprod*. 2012;27:2908–17.
20. Zhao J, Zhang Q, Wang Y, et al. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: A systematic review and meta-analysis. *Fertil Steril*. 2014;102: 998–1005.
21. Carlini T, Paoli D, Pelloni M, et al. Sperm DNA fragmentation in Italian couples with recurrent pregnancy loss. *Reprod Biomed Online*. 2017;34:58–65.
22. Haddock L, Gordon S, Lewis SEM, et al. Sperm DNA fragmentation is a novel biomarker for early pregnancy loss. *Reprod Biomed Online*. 2021;42:175–84.
23. Simon L, Zini A, Dyachenko A, et al. A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. *Asian J Androl*. 2017;19:80–90.
24. Greco E, Romano S, Iacobelli M, et al. ICSI in cases of sperm DNA damage: Beneficial effect of oral antioxidant treatment. *Hum Reprod*. 2005;20:2590–4.
25. Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? *Fertil Steril*. 2008;89:823–31.
26. Practice Committee of the American Society for Reproductive Medicine. The clinical utility of sperm DNA integrity testing: A guideline. *Fertil Steril*. 2013;99:673–7.
27. Jarow J, Sigman M, Kolettis P. The Optimal Evaluation of the Infertile Male: Best Practice Statement Reviewed and Validity Confirmed. American Urological Association, 2011. <https://www.auanet.org/guidelines/male-infertility-optimal-evaluation-best-practice-statement>
28. Jungwirth A, Diemer T, Dohle G, et al. Guidelines on Male Infertility, European Association of Urology Guidelines. Arnhem the Netherlands, 2015. https://uroweb.org/wp-content/uploads/17-Male-Infertility_LR1.pdf
29. Practice Committee of the American Society for Reproductive Medicine. Diagnostic evaluation of the infertile male: A committee opinion. *Fertil Steril*. 2015;103:e18–25.
30. Agarwal A, Panner Selvam MK, Baskaran S, et al. Sperm DNA damage and its impact on male reproductive health: A critical review for clinicians, reproductive professionals and researchers. *Expert Rev Mol Diagn*. 2019;6:443–57.
31. Agarwal A, Majzoub A, Baskaran S, et al. Sperm DNA fragmentation: A new guideline for clinicians. *World J Mens Health*. 2020;38:412–71.
32. Agarwal A, Farkouh A, Parekh N, et al. Sperm DNA fragmentation: A critical assessment of clinical practice guidelines. *World J Mens Health*. 2022;40:30–7.
33. Deng C, Li T, Xie Y, et al. Sperm DNA fragmentation index influences assisted reproductive technology outcome: A systematic review and meta-analysis combined with a retrospective cohort study. *Andrologia*. 2019;51:e13263.
34. Esteves SC, Agarwal A, Cho CL, Majzoub A. A Strengths-Weaknesses-Opportunities-Threats (SWOT) analysis on the clinical utility of sperm DNA fragmentation testing in specific male infertility scenarios. *Transl Androl Urol*. 2017b;6:S734–S760.
35. Esteves SC, Zini A, Coward RM, et al. Sperm DNA fragmentation testing: Summary evidence and clinical practice recommendations. *Andrologia*. 2021;53:e13874.
36. Tharakan T, Bettocchi C, Carvalho J, et al. European Association Of Urology Guidelines Panel on male sexual and reproductive health: A clinical consultation guide on the indications for performing sperm DNA fragmentation testing in men with infertility and testicular sperm extraction in nonazoospermic men. *Eur Urol Focus*. 2022;8:339–50.
37. Agarwal A, Cho CL, Majzoub A, et al. Call for wider application of sperm DNA fragmentation test. *Transl Androl Urol*. 2017a;6:S399–01.
38. Agarwal A, Cho CL, Majzoub A, et al. Risk factors associated with sperm DNA fragmentation. *Transl Androl Urol*. 2017b;6:S519–21.
39. Cho CL, Esteves SC, Agarwal A. Novel insights into the pathophysiology of varicocele and its association with reactive oxygen species and sperm DNA fragmentation. *Asian J Androl*. 2016;18:186–93.
40. Roque M, Esteves SC. Effect of varicocele repair on sperm DNA fragmentation: A review. *Int Urol Nephrol*. 2018;50:583–603.
41. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen, 6th Edition. Geneva: World Health Organization, 2021.
42. Bellve AR, McKay DJ, Renaux BS, et al. Purification and characterization of mouse protamines P1 and P2. Amino acid sequence of P2. *Biochem*. 1988;27:2890–7.
43. Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells. *Biol Reprod*. 1991;44:569–74.
44. Fuentes-Mascorro G, Serrano H, Rosado A. Sperm chromatin. *Arch Androl*. 2000;45:215–25.
45. Oliva R, Castillo J. Proteomics and the genetics of sperm chromatin condensation. *Asian J Androl*. 2011;13:24–30.
46. Gatewood JM, Cook GR, Balhorn R, et al. Sequence-specific packaging of DNA in human sperm chromatin. *Science*. 1987;236:962–4.
47. Bench GS, Friz AM, Corzett MH, et al. DNA and total protamine masses in individual sperm from fertile mammalian subjects. *Cytometry*. 1996;23:263–71.
48. Johnson GD, Lalancette C, Linnemann AK, et al. The sperm nucleus: Chromatin, RNA, and the nuclear matrix. *Reproduction*. 2011;141:21–36.
49. Sakkas D, Mariethoz E, Manicardi G, et al. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod*. 1999;4:31–7.

50. Ward WS. Deoxyribonucleic acid loop domain tertiary structure in mammalian spermatozoa. *Biol Reprod.* 1993;48:1193–201.
51. Zalensky AO, Allen MJ, Kobayashi A, et al. Well-defined genome architecture in the human sperm nucleus. *Chromosoma.* 1995;103:577–90.
52. Solov'eva L, Svetlova M, Bodinski D, et al. Nature of telomere dimers and chromosome looping in human spermatozoa. *Chromosome Res.* 2004;12:817–23.
53. Ward WS, Zalensky AO. The unique, complex organization of the transcriptionally silent sperm chromatin. *Crit Rev Eukaryot Gene Expr.* 1996;6:139–47.
54. Lewis JD, Song Y, de Jong ME, et al. A walk through vertebrate and invertebrate protamines. *Chromosoma.* 1999;111:473–82.
55. Corzett M, Mazrimas J, Balhorn R. Protamine 1: Protamine 2 stoichiometry in the sperm of eutherian mammals. *Mol Reprod Dev.* 2002;61:519–27.
56. Jager S. Sperm nuclear stability and male infertility. *Arch Androl.* 1990;25:253–9.
57. Torregrosa N, Domínguez-Fandos D, Camejo MI, et al. Protamine 2 precursors, protamine 1/protamine 2 ratio, DNA integrity and other sperm parameters in infertile patients. *Hum Reprod.* 2006;21:2084–9.
58. Steger K, Wilhelm J, Konrad L, et al. Both protamine-1 to protamine-2 mRNA ratio and Bcl2 mRNA content in testicular spermatids and ejaculated spermatozoa discriminate between fertile and infertile men. *Hum Reprod.* 2008;23:11–6.
59. Balhorn R, Reed S, Tanphaichitr N. Aberrant protamine 1/protamine 2 ratio in sperm of infertile human males. *Experientia.* 1988;44:52–5.
60. Bench G, Corzett MH, De Yebra L, et al. Protein and DNA contents in sperm from an infertile human male possessing protamine defects that vary over time. *Mol Reprod Dev.* 1998;50:345–53.
61. de Yebra L, Ballesca JL, Vanrell JA, et al. Detection of P2 precursors in the sperm cells of infertile patients who have reduced protamine P2 levels. *Fertil Steril.* 1998;69:755–9.
62. Aoki VW, Liu L, Carrell DT. Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. *Hum Reprod.* 2005;20:1298–306.
63. Oliva R. Protamines and male infertility. *Hum Reprod Update.* 2006;12:417–35.
64. Sarasa J, Enciso M, García L, et al. Comparison of ART outcomes in men with altered mRNA protamine 1/protamine 2 ratio undergoing intracytoplasmic sperm injection with ejaculated and testicular spermatozoa. *Asian J Androl.* 2020;22:623–8.
65. Amjad S, Mushtaq S, Rehman R, et al. Protamine 1/Protamine 2 mRNA ratio in nonobstructive azoospermic patients. *Andrologia.* 2021;53:e13936.
66. Simon L, Castillo J, Oliva R, et al. Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. *Reprod Biomed Online.* 2011;23:724–34.
67. Marchiani S, Tamburrino L, Benini F, et al. Chromatin protamination and CatSper expression in spermatozoa predict clinical outcomes after assisted reproduction programs. *Sci Rep.* 2017;7:15122.
68. García-Péiró A, Martínez-Heredia J, Oliver-Bonet M, et al. Protamine 1 to protamine 2 ratio correlates with dynamic aspects of DNA fragmentation in human sperm. *Fertil Steril.* 2011;95:105–9.
69. Nanassy L, Liu L, Griffin J, et al. The clinical utility of the protamine 1/protamine 2 ratio in sperm. *Protein Pept Lett.* 2011;18:772–7.
70. Matsuda Y, Tobari I. Chromosomal analysis in mouse eggs fertilized *in vitro* with sperm exposed to ultraviolet light (UV) and methyl and ethyl methanesulfonate (MMS and EMS). *Mutat Res.* 1988;198:131–44.
71. Genesca A, Caballin MR, Miro R, et al. Repair of human sperm chromosome aberrations in the hamster egg. *Hum Genet.* 1992;89:181–6.
72. Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction.* 2001;122:497–506.
73. Muratori M, Tamburrino L, Marchiani S, et al. Investigation on the origin of sperm DNA fragmentation: Role of apoptosis, immaturity and oxidative stress. *Mol Med.* 2015;21:109–22.
74. Sakkas D, Manicardi G, Bianchi PG, et al. Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. *Biol Reprod.* 1995;52:1149–55.
75. Marcon L, Boissonneault G. Transient DNA strand breaks during mouse and human spermiogenesis: New insights in stage specificity and link to chromatin remodeling. *Biol Reprod.* 2004;70:910–18.
76. Finelli R, Leisegang K, Kandil H, et al. Oxidative stress: A comprehensive review of biochemical, molecular, and genetic aspects in the pathogenesis and management of varicocele. *World J Mens Health.* 2022;40:87–103.
77. McPherson SM, Longo FJ. Localization of DNase I-hypersensitive regions during rat spermatogenesis: Stage-dependent patterns and unique sensitivity of elongating spermatids. *Mol Reprod Dev.* 1992;31:268–79.
78. Laberge RM, Boissonneault G. On the nature and origin of DNA strand breaks in elongating spermatids. *Biol Reprod.* 2005;73:289–96.
79. Kierszenbaum AL. Transition nuclear proteins during spermiogenesis: Unrepaired DNA breaks not allowed. *Mol Reprod Dev.* 2001;58:357–8.
80. Balhorn R, Cosman M, Thornton K, et al. Protamine mediated condensation of DNA in mammalian sperm. In: *The Male Gamete: From Basic Knowledge to Clinical Applications.* Gagnon C (ed.). Vienna, IL: Cache River Press, pp. 55–70, 1999.
81. Morse-Gaudio M, Risley MS. Topoisomerase II expression and VM-26 induction of DNA breaks during spermatogenesis in *Xenopus laevis*. *J Cell Sci.* 1994;107:2887–98.
82. Bizzaro D, Manicardi G, Bianchi PG, et al. Sperm decondensation during fertilisation in the mouse: Presence of DNase I hypersensitive sites *in situ* and a putative role for topoisomerase II. *Zygote.* 2000;8:197–202.
83. Boitrelle F, Ferfouri F, Petit JM, et al. Large human sperm vacuoles observed in motile spermatozoa under high magnification: Nuclear thumbprints linked to failure of chromatin condensation. *Hum Reprod.* 2011;26:1650–8.
84. Franco JG Jr, Mauri AL, Petersen CG, et al. Large nuclear vacuoles are indicative of abnormal chromatin packaging in human spermatozoa. *Int J Androl.* 2012;35:46–51.
85. Bendayan M, Caceres L, Sais E, Swierkowski-Blanchard N, Alter L, Bonnet-Garnier A, Boitrelle F. Human sperm morphology as a marker of its nuclear quality and epigenetic pattern. *Cells.* 2022;11:1788.
86. Aoki VW, Liu L, Jones KP, et al. Sperm protamine1/protamine 2 ratios are related to *in vitro* fertilization pregnancy rates and predictive of fertilization ability. *Fertil Steril.* 2006;86:1408–15.
87. Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished spermato-genesis: What is the link? *Hum Reprod Update.* 2007;13:313–27.
88. Tseden K, Topaloglu O, Meinhardt A, et al. Premature translation of transition protein 2 mRNA causes sperm abnormalities and male infertility. *Mol Reprod Dev.* 2007;74:273–9.
89. Sakkas D, Mariethoz E, St John JC. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp Cell Res.* 1999;251:350–5.
90. Donnelly ET, O'Connell M, McClure N, et al. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. *Hum Reprod.* 2000;15:1552–61.
91. Spadafora C. Sperm cells and foreign DNA: A controversial relation. *Bioessays.* 1998;20:955–64.
92. Aitken RJ, Koppers AJ. Apoptosis and DNA damage in human spermatozoa. *Asian J Androl.* 2011;13:36–42.
93. Suda T, Takahashi T, Golstein P, et al. Molecular cloning and expression of the fas ligand, a novel member of the tumor necrosis factor family. *Cell.* 1993;75:1169–78.

94. Rodriguez I, Ody C, Araki K, et al. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J.* 1997;16:2262–70.
95. Said TM, Paasch U, Glander HJ, et al. Role of caspases in male infertility. *Hum Reprod Update.* 2004;10:39–51.
96. Zhivotovsky B, Kroemer G. Apoptosis and genomic instability. *Nat Rev Mol Cell Biol.* 2004;5:752–62.
97. Muratori M, Piomboni P, Baldi E, et al. Functional and ultrastructural features of DNA-fragmented human sperm. *J Androl.* 2000;21:903–12.
98. Barroso G, Morshed M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod.* 2000;15:1338–44.
99. Ross AJ, Waymire KG, Moss JE, et al. Testicular degeneration in Bclw-deficient mice. *Nat Genet.* 1998;18:251–6.
100. Sakkas D, Moffatt O, Manicardi GC, et al. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod.* 2002;66:1061–7.
101. Sutovsky P, Neuber E, Schatten G. Ubiquitin dependent sperm quality control mechanism recognizes spermatozoa with DNA defects as revealed by dual ubiquitin-TUNEL assay. *Mol Reprod Dev.* 2002;61:406–13.
102. Muratori M, Maggi M, Spinelli S, et al. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl.* 2003;24:253–62.
103. Sakkas D, Seli E, Bizzaro D, et al. Abnormal spermatozoa in the ejaculate: Abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online.* 2003;7:428–32.
104. Henkel R, Hajimohammad M, Stalf T, et al. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril.* 2004;81:965–72.
105. Lachaud C, Tesarik J, Canadas ML, et al. Apoptosis and necrosis in human ejaculated spermatozoa. *Hum Reprod.* 2004;19:607–10.
106. Moustafa MH, Sharma RK, Thornton J, et al. Relationship between ROS production, apoptosis, and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod.* 2004;19:129–38.
107. Paasch U, Sharma RK, Gupta AK, et al. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod.* 2004;71:1828–37.
108. Sutovsky P, Hauser R, Sutovsky M. Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology clinic population. *Hum Reprod.* 2004;19:628–35.
109. Aitken RJ, Buckingham D, West K, et al. Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. *J Reprod Fertil.* 1992;94:451–62.
110. Tremellen K. Oxidative stress and male infertility—a clinical perspective. *Hum Reprod Update.* 2008;14:243–58.
111. Bisht S, Faiq M, Tolahunase M, et al. Oxidative stress and male infertility. *Nat Rev Urol.* 2017;14:470–85.
112. Agarwal A, Rana M, Qiu E, et al. Role of oxidative stress, infection and inflammation in male infertility. *Andrologia.* 2018;11:e13126.
113. Aitken RJ, Baker MA. The role of genetics and oxidative stress in the etiology of male infertility – A unifying hypothesis? *Front Endocrinol (Lausanne).* 2020;11:581838.
114. Ritchie C, Ko EY. Oxidative stress in the pathophysiology of male infertility. *Andrologia.* 2021;53:e13581.
115. Dutta S, Majzoub A, Agarwal A. Oxidative stress and sperm function: A systematic review on evaluation and management. *Arab J Urol.* 2019;17:87–97.
116. Martínez P, Proverbio F, Camejo MI. Sperm lipid peroxidation and pro-inflammatory cytokines. *Asian J Androl.* 2007;9:102–7.
117. Humaidan P, Haahr T, Povlsen BB, et al. The combined effect of lifestyle intervention and antioxidant therapy on sperm DNA fragmentation and seminal oxidative stress in IVF patients: A pilot study. *Int Braz J Urol.* 2022;48:131–56.
118. Vaughan DA, Tirado E, Garcia D, et al. DNA fragmentation of sperm: A radical examination of the contribution of oxidative stress and age in 16945 semen samples. *Hum Reprod.* 2020;35:2188–96.
119. Gosálvez J, López-Fernández C, Fernandez JL et al. Unpacking the mysteries of sperm DNA fragmentation: Ten frequently asked questions. *J Reprod Biotech Fertil.* 2015; 31: 2058915815594454.
120. Ribas-Maynou J, Benet J. Single and double strand sperm DNA damage: Different reproductive effects on male fertility. *Genes (Basel).* 2019;10:105.
121. Bannister LA, Schimenti JC. Homologous recombinational repair proteins in mouse meiosis. *Cytogenet Genome Res.* 2004;107:191–200.
122. De Iuliis GN, Thomson LK, Mitchell LA, et al. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodelling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod.* 2009;81:517–24.
123. Palmer NO, Bakos HW, Fullston T, et al. Impact of obesity on male fertility, sperm function and molecular composition. *Spermatogenesis.* 2012;2:253–63.
124. Agarwal A, Deepinder F, Sharma RK, et al. Effect of cell phone usage on semen analysis in men attending infertility clinic: An observational study. *Fertil Steril.* 2008;89:124–8.
125. Dupont C, Faure C, Sermondade N, et al. Obesity leads to higher risk of sperm DNA damage in infertile patients. *Asian J Androl.* 2013;15:622.
126. Harley A, Agarwal A, Gunes SO, et al. Smoking and male infertility: An evidence-based review. *World J Mens Health.* 2015;33:143–60.
127. Gavrilouk D, Aitken RJ. Damage to sperm DNA mediated by reactive oxygen species: Its impact on human reproduction and the health trajectory of offspring. *Adv Exp Med Biol.* 2015;868:23–47.
128. Singh NP, Muller CH, Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. *Fertil Steril.* 2003;80:1420–30.
129. Schmid TE, Eskenazi B, Baumgartner A, et al. The effects of male age on sperm DNA damage in healthy non-smokers. *Hum Reprod.* 2007;22:180–7.
130. Alshahrani S, Agarwal A, Assidi M, et al. Infertile men older than 40 years are at higher risk of sperm DNA damage. *Reprod Biol Endocrinol.* 2014;12:103.
131. Sharma R, Agarwal A, Rohra VK, et al. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reprod Biol Endocrinol.* 2015;13:35.
132. Morris ID. Sperm DNA damage and cancer treatment. *Int J Androl.* 2002;25:255–61.
133. Sailer BL, Jost LK, Erickson KR, et al. Effects of X-irradiation on mouse testicular cells and sperm chromatin structure. *Environ Mol Mutagen.* 1995;25:23–30.
134. Fossa SD, De Angelis P, Krägerud SM, et al. Prediction of post-treatment spermatogenesis in patients with testicular cancer by flow cytometric sperm chromatin structure assay. *Cytometry.* 1997;30:192–6.
135. Ståhl O, Eberhard J, Jepson K, et al. Sperm DNA integrity in testicular cancer patients. *Hum Reprod.* 2006;21:3199–205.
136. Saleh RA, Agarwal A, Sharma RK, et al. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: A prospective study(2002). *Fertil Steril.* 2002;78:491–9.
137. Erenpreiss J, Hlevicka S, Zalkalns J, et al. Effect of leukocytospermia on sperm DNA integrity: A negative effect in abnormal semen samples. *J Androl.* 2002;23:717–23.
138. Rubes J, Selevan SG, Evenson DP, et al. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. *Hum Reprod.* 2005;20:2776–83.
139. Lewis SEM, Esteves SC. What does a varicocele do to a man's fertility? There is much more than meets the eye. *Int Braz J Urol.* 2021;47:284–6.

140. Saleh RA, Agarwal A, Sharma RK, et al. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. *Fertil Steril.* 2003;80:1431–6.
141. Zini A, Defreitas G, Freeman M, et al. Varicocele is associated with abnormal retention of cytoplasmic droplets by human spermatozoa. *Fertil Steril.* 2000;74:461–4.
142. Zini A, Dohle G. Are varicoceles associated with increased deoxyribonucleic acid fragmentation? *Fertil Steril.* 2011;96:1283–7.
143. Esteves SC, Santi D, Simoni M. An update on clinical and surgical interventions to reduce sperm DNA fragmentation in infertile men. *Andrology.* 2020;8:53–81.
144. Panner Selvam MK, Samanta L, Agarwal A. Functional analysis of differentially expressed acetylated spermatozoal proteins in infertile men with unilateral and bilateral varicocele. *Int J Mol Sci.* 2020;21:3155.
145. Ammar O, Tekeya O, Hannachi I, et al. Increased sperm DNA fragmentation in infertile men with varicocele: Relationship with apoptosis, seminal oxidative stress, and spermatic parameters. *Reprod Sci.* 2021;28:909–19.
146. Finelli R, Darbandi S, Pushparaj PN, et al. Silico sperm proteome analysis to investigate DNA repair mechanisms in varicocele patients. *Front Endocrinol (Lausanne).* 2021;12:757592.
147. Finelli R, Moreira BP, Alves MG, Agarwal A. Unraveling the Molecular Impact of Sperm DNA Damage on Human Reproduction. *Adv Exp Med Biol.* 2022;1358:77–113.
148. Jeremias JT, Belardin LB, Okada FK, et al. Oxidative origin of sperm DNA fragmentation in the adult varicocele. *Int Braz J Urol.* 2021;47:275–83.
149. Zini A, Blumenfeld A, Libman J, et al. Beneficial effect of micro-surgical varicocelectomy on human sperm DNA integrity. *Hum Reprod.* 2005;20:1018–21.
150. Smit M, Romijn JC, Wildhagen MF, et al. Decreased sperm DNA fragmentation after surgical varicocelectomy is associated with increased pregnancy rate. *J Urol.* 2010;183:270–4.
151. Wang YJ, Zhang RQ, Lin YJ, et al. Relationship between varicocele and sperm DNA damage and the effect of varicocele repair: A meta-analysis. *Reprod Biomed Online.* 2012;25:307–14.
152. Lira Neto FT, Roque M, Esteves SC. Effect of varicocelectomy on sperm deoxyribonucleic acid fragmentation rates in infertile men with clinical varicoceles: A systematic review and meta-analysis. *Fertil Steril.* 2021;116:696–712.
153. Wang SL, Bedrick BS, Kohn TP. What is the role of varicocelectomy in infertile men with clinical varicoceles and elevated sperm DNA fragmentation? *Fertil Steril.* 2021;116:657–8.
154. Qiu D, Shi Q, Pan L. Efficacy of varicocelectomy for sperm DNA integrity improvement: A meta-analysis. *Andrologia.* 2021;53:e13885.
155. Xing W, Krishnamurthy H, Sairam MR. Role of follitropin receptor signaling in nuclear protein transitions and chromatin condensation during spermatogenesis. *Biochem Biophys Res Commun.* 2003;312:697–701.
156. Evenson DP, Jost LK, Corzett M, et al. Characteristics of human sperm chromatin structure following an episode of influenza and high fever: A case study. *J Androl.* 2000;21:739–46.
157. Ahmad G, Moinard N, Esquerre-Lamare C, et al. Mild induced testicular and epididymal hyperthermia alters sperm chromatin integrity in men. *Fertil Steril.* 2012;97:546–53.
158. Cho CL, Agarwal A, Majzoub A, et al. Clinical utility of sperm DNA fragmentation testing: Concise practice recommendations. *Transl Androl Urol.* 2017;6:S366–73.
159. Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros and cons. *J Androl.* 2009;30:219–29.
160. Bareh GM, Jacoby E, Binkley P, et al. Sperm deoxyribonucleic acid fragmentation assessment in normozoospermic male partners of couples with unexplained recurrent pregnancy loss: A prospective study. *Fertil Steril.* 2016;105:329–36.
161. O'Neill CL, Parrella A, Keating D, et al. A treatment algorithm for couples with unexplained infertility based on sperm chromatin assessment. *J Assist Reprod Genet.* 2018;35:1911–7.
162. Majzoub A, Agarwal A, Cho CL, et al. Sperm DNA fragmentation testing: A cross sectional survey on current practices of fertility specialists. *Transl Androl Urol.* 2017;6:S710–19.
163. Sharma R, Ahmad G, Esteves S, et al. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using bench top flow cytometer for evaluation of sperm DNA fragmentation in fertility laboratories: Protocol, reference values, and quality control. *J Assist Reprod Genet.* 2016;33:291–300.
164. Sergerie M, Laforest G, Boulanger K, et al. Longitudinal study of sperm DNA fragmentation as measured by terminal uridine nick end-labelling assay. *Hum Reprod.* 2005;20:1921–7.
165. Erenpreiss J, Bungum M, Spano M, et al. Intra-individual variation in sperm chromatin structure assay parameters in men from infertile couples: Clinical implications. *Hum Reprod.* 2006;21:2061–4.
166. Smit M, Dohle GR, Hop WC, et al. Clinical correlates of the biological variation of sperm DNA fragmentation in infertile men attending an andrology outpatient clinic. *Int J Androl.* 2007;30:48–55.
167. Spano M, Bonde JP, Hjollund HI, et al. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. *Fertil Steril.* 2000;73:43–50.
168. Gandini L, Lombardo F, Paoli D, et al. Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod.* 2000;15:830–9.
169. Larson-Cook KL, Brannian JD, Hansen KA, et al. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril.* 2003;80:895–902.
170. Saleh RA, Agarwal A, Nelson DR, et al. Increased sperm nuclear DNA damage in normozoospermic infertile men: A prospective study. *Fertil Steril.* 2002;78:313–8.
171. Zini A, Bielecki R, Phang D, et al. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril.* 2001;75:674–7.
172. McKelvey-Martin V, Melia N, Walsh I, et al. Two potential clinical applications of the alkaline single-cell gel electrophoresis assay: (1). Human bladder washings and transitional cell carcinoma of the bladder; and (2). Human sperm and male infertility. *Mutat Res.* 1997;375:93–104.
173. Zhang Y, Wang H, Wang L, et al. The clinical significance of sperm DNA damage detection combined with routine semen testing in assisted reproduction. *Mol Med Report.* 2008;1:617–24.
174. Sergerie M, Laforest G, Bujan L, et al. Sperm DNA fragmentation: Threshold value in male fertility. *Hum Reprod.* 2005;20:3446–51.
175. Drobnis EZ, Johnson M. The question of sperm DNA fragmentation testing in the male infertility work-up: A response to Professor Lewis' commentary. *Reprod Biomed Online.* 2015;31:138–9.
176. Saleh RA, Agarwal A, Nada ES, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril.* 2003;79:1597–605.
177. Bungum M, Humaidan P, Spano M, et al. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod.* 2004;19:1401–8.
178. Duran EH, Morshed M, Taylor S, et al. Sperm DNA quality predicts intrauterine insemination outcome: A prospective cohort study. *Hum Reprod.* 2002;17:3122–8.
179. Simon L, Carrell DT, Zini A. Sperm DNA Tests Are Clinically Useful: Pro. A Clinician's Guide to Sperm DNA and Chromatin Damage. Cham: Springer, pp. 431–67, 2018.
180. Agarwal A, Cho C-L, Esteves SC. Should we evaluate and treat sperm DNA fragmentation? *Curr Opin Obstet Gynecol.* 2016;28:164–71.
181. Payne JF, Raburn DJ, Couchman GM, et al. Redefining the relationship between sperm deoxyribonucleic acid fragmentation as measured by the sperm chromatin structure assay and outcomes of assisted reproductive techniques. *Fertil Steril.* 2005;84:356–64.

182. Tomlinson MJ, Moffatt O, Manicardi GC, et al. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: Implications for assisted conception. *Hum Reprod.* 2001;16:2160–5.
183. Seli E, Gardner DK, Schoolcraft WB, et al. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after *in vitro* fertilization. *Fertil Steril.* 2004;82:378–83.
184. Hammadah ME, Stieber M, Haidl G, et al. Association between sperm cell chromatin condensation, morphology based on strict criteria, and fertilization, cleavage and pregnancy rates in an IVF program. *Andrologia.* 1998;30:29–35.
185. Lopes S, Sun JG, Jurisicova A, et al. Sperm deoxyribonucleic acid fragmentation is increased in poor quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril.* 1998;69:528–32.
186. Twigg JP, Irvine DS, Aitken RJ. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum Reprod.* 1998;13:1864–71.
187. Li Z, Wang L, Cai J, et al. Correlation of sperm DNA damage with IVF and ICSI outcomes: A systematic review and meta-analysis. *J Assist Reprod Genetics.* 2006;23:367–76.
188. Rilcheva VS, Ayvazova NP, Ilieva LO, et al. Sperm DNA integrity test and assisted reproductive technology (art) outcome. *J Biomed Clin Res.* 2016;9:21.
189. Zhang Z, Dai C, Shan G, Chen X, Liu H, Abdalla K, Kuznyetsova I, Moskovstev S, Huang X, Librach C, Jarvi K, Sun Y. Quantitative selection of single human sperm with high DNA integrity for intracytoplasmic sperm injection. *Fertil Steril.* 2021;116:1308–1318.
190. Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: A clinical approach. *BJU Int.* 2005;95:503–7.
191. Aziz N, Said T, Paasch U, et al. The relationship between human sperm apoptosis, morphology and the sperm deformity index. *Hum Reprod.* 2007;22:1413–9.
192. Khadem N, Poorhoseyni A, Jalali M, et al. Sperm DNA fragmentation in couples with unexplained recurrent spontaneous abortions. *Andrologia.* 2014;46:126–30.
193. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril.* 2004;81:1289–95.
194. Paoli D, Pallotti F, Lenzi A, et al. Fatherhood and sperm DNA damage in testicular cancer patients. *Front Endocrinol (Lausanne).* 2018;9:506.
195. Bujan L, Walschaerts M, Brugnon F, et al. Impact of lymphoma treatments on spermatogenesis and sperm deoxyribonucleic acid: A multicenter prospective study from the CECOS network. *Fertil Steril.* 2014;102:667–74.
196. Daudin M, Rives N, Walschaerts M, et al. Sperm cryopreservation in adolescents and young adults with cancer: Results of the French national sperm banking network (CECOS). *Fertil Steril.* 2015;103:478–86.
197. De Mas P, Daudin M, Vincent MC, et al. Increased aneuploidy in spermatozoa from testicular tumour patients after chemotherapy with cisplatin, etoposide and bleomycin. *Hum Reprod.* 2001;16:1204–8.
198. Kobayashi H, Larson K, Sharma R, et al. DNA damage in cancer patients before treatment as measured by the sperm chromatin structure assay. *Fertil Steril.* 2001;75:469–75.
199. O'Flaherty C, Vaisheva F, Hales BF, et al. Characterization of sperm chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to chemotherapy. *Hum Reprod.* 2008;23:1044–52.
200. Smit M, Van Casteren N, Wildhagen M, et al. Sperm DNA integrity in cancer patients before and after cytotoxic treatment. *Hum Reprod.* 2010;25:1877–83.
201. Beaud H, Tremblay AR, Chan PTK, et al. Sperm DNA damage in cancer patients. *Adv Exp Med Biol.* 2019;1166:189–203.
202. Parekh NV, Lundy SD, Vij SC. Fertility considerations in men with testicular cancer. *Transl Androl Urol.* 2020;9:S14–23.
203. García A, Herrero MB, Holzer H, et al. Assisted reproductive outcomes of male cancer survivors. *J Cancer Surviv.* 2015;9:208–14.
204. Papler TB, Vrtacnik-Bokal E, Drobnič S, et al. The outcome of IVF/ICSI cycles in male cancer patients: Retrospective analysis of procedures from 2004 to 2018. *Radiol Oncol.* 2021;55:221–8.
205. Baker H, Liu D. Assessment of nuclear maturity. In: *Human Spermatozoa in Assisted Reproduction*. Acosta A, Kruger T (eds). London: CRC Press, pp. 193–203, 1996.
206. Erenpreisa J, Erenpreiss J, Freivalds T, et al. Toluidine blue test for sperm DNA integrity and elaboration of image cytometry algorithm. *Cytometry.* 2003;52:19–27.
207. Manicardi GC, Bianchi PG, Pantano S, et al. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A₃ accessibility. *Biol Reprod.* 1995;52:864–7.
208. Fernandez JL, Vazquez-Gundin F, DelgFernando A, et al. DNA breakage detection–FISH (DBD–FISH) in human spermatozoa: Technical variants evidence different structural features. *Mutat Res.* 2000;453:77–82.
209. Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* 1993;53:1945–51. [PMC]8467513
210. Zini A, Fischer MA, Sharir S, et al. Prevalence of abnormal sperm DNA denaturation in fertile and infertile men. *Urology.* 2002;60:1069–72.
211. Fernandez JL, Muriel L, Rivero MT, et al. The sperm chromatin dispersion test: A simple method for the determination of sperm DNA fragmentation. *J Androl.* 2003;24:59–66.
212. Singh NP, McCoy MT, Tice RR, et al. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988;175:184–91.
213. Singh NP, Danner DB, Tice RR. Abundant alkali-sensitive sites in DNA of human and mouse sperm. *Exp Cell Res.* 1989;184:461–70.
214. Ribeiro S, Sharma R, Gupta S, et al. Inter- and intra-laboratory standardization of TUNEL assay for assessment of sperm DNA fragmentation. *Andrology.* 2017;5:477–85.
215. Gupta S, Sharma R, Agarwal A. Inter- and intra-laboratory standardization of TUNEL assay for assessment of sperm DNA fragmentation. *Curr Protoc Toxicol.* 2017;74:1–16.
216. Sharma R, Gupta S, Henkel R, et al. Critical evaluation of two models of flow cytometers for the assessment of sperm DNA fragmentation: An appeal for performance verification. *Asian J Androl.* 2019;21:438–44.
217. Kodama H, Yamaguchi R, Fukuda J. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril.* 1997;68:519–24.
218. Shen H, Ong C. Detection of oxidative DNA damage in human sperm and its association with sperm function and male infertility. *Free Radic Biol Med.* 2000;28:529–36.
219. Hammadah ME, Zeginiadov T, Rosenbaum P, et al. Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. *Arch Androl.* 2001;46:99–104.
220. Wong A, Chuan SS, Patton WC, et al. Addition of eosin to the aniline blue assay to enhance detection of immature sperm histones. *Fertil Steril.* 2008;90:1999–2002.
221. Foresta C, Zorzi M, Rossato M, et al. Sperm nuclear instability and staining with aniline blue: Abnormal persistence of histones in spermatozoa in infertile men. *Int J Androl.* 1992;15:330–7.
222. Auger J, Mesbah M, Huber C, et al. Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. *Int J Androl.* 1990;13:452–62.
223. Kazerooni T, Asadi N, Jadid L, et al. Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A₃ and aniline blue staining in couples with unexplained recurrent abortion. *J Assist Reprod Genet.* 2009;26:591–6.

224. de Jager C, Aneck-Hahn NH, Bornman MS, et al. Sperm chromatin integrity in DDT-exposed young men living in a malaria area in the Limpopo Province, South Africa. *Hum Reprod.* 2009;24: 2429–38.
225. Erenpreiss J, Bars J, Lipatnikova V, et al. Comparative study of cytochemical tests for sperm chromatin integrity. *J Androl.* 2001;22:45–53.
226. Erenpreiss J, Jepson K, Giwercman A, et al. Toluidine blue cytometry test for sperm DNA conformation: Comparison with the flow cytometric sperm chromatin structure and TUNEL assays. *Hum Reprod.* 2004;19:2277–82.
227. Tsarev I, Bungum M, Giwercman A, et al. Evaluation of male fertility potential by Toluidine Blue test for sperm chromatin structure assessment. *Hum Reprod.* 2009;24:1569–74.
228. Sadeghi MR, Lakpour N, Heidari-Vala H, et al. Relationship between sperm chromatin status and ICSI outcome in men with obstructive azoospermia and unexplained infertile normozoospermia. *Rom J Morphol Embryol.* 2011;52:645–51.
229. Carretero MI, Giuliano SM, Casaretto CI, et al. Evaluation of the effect of cooling and of the addition of collagenase on llama sperm DNA using toluidine blue. *Andrologia.* 2011;44:239–47.
230. Talebi AR, Moein MR, Tabibnejad N, et al. Effect of varicocele on chromatin condensation and DNA integrity of ejaculated spermatozoa using cytochemical tests. *Andrologia.* 2008;40:245–51.
231. Mahfouz RZ, Sharma RK, Said TM, et al. Association of sperm apoptosis and DNA ploidy with sperm chromatin quality in human spermatozoa. *Fertil Steril.* 2009;91:1110–8.
232. Hosseinifar H, Yazdanikhah S, Modarresi T, et al. Correlation between sperm DNA fragmentation index and CMA3 positive spermatozoa in globozoospermic patients. *Andrology.* 2015;3:526–31.
233. Esterhuizen ad, Franken DR, Lourens JG, et al. Sperm chromatin packaging as an indicator of *in-vitro* fertilization rates. *Hum Reprod.* 2000;15:657–61.
234. Sakkas D, Urner F, Bianchi PG, et al. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Hum Reprod.* 1996;11:837–43.
235. Sakkas D, Urner F, Bizzaro D, et al. Sperm nuclear DNA damage and altered chromatin structure: Effect on fertilization and embryo development. *Hum Reprod.* 1998;13:11–9.
236. Nijls M, Creemers E, Cox A, et al. Chromomycin A₃ staining, sperm chromatin structure assay and hyaluronic acid binding assay as predictors for assisted reproductive outcome. *Reprod Biomed Online.* 2009;19:671–84.
237. Manochantr S, Chiamchanya C, Sobhon P. Relationship between chromatin condensation, DNA integrity and quality of ejaculated spermatozoa from infertile men. *Andrologia.* 2012;44:187–99.
238. Fernandez JL, Goyanes VJ, Ramiro-Diaz J, et al. Application of FISH for *in situ* detection and quantification of DNA breakage. *Cytogenet Cell Genet.* 1998;82:251–6.
239. Fernández JL, Cajigal D, Gosálvez J. Simultaneous labeling of single- and double-strand DNA breaks by DNA breakage detection–FISH (DBD–FISH). *Methods Mol Biol.* 2011;682:133–47.
240. Irvine DS, Twigg JP, Gordon EL, et al. DNA integrity in human spermatozoa: Relationships with semen quality. *J Androl.* 2000;21:33–44.
241. Shamsi MB, Kumar R, Dada R. Evaluation of nuclear DNA damage in human spermatozoa in men opting for assisted reproduction. *Indian J Med Res.* 2008;127:115–23.
242. Setchell BP, Ekpe G, Zupp JL, et al. Transient retardation in embryo growth in normal female mice made pregnant by males whose testes had been heated. *Hum Reprod.* 1998;13:342–7.
243. Aitken RJ, Irvine DS, Wu FC. Prospective analysis of sperm–oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol.* 1991;164: 542–51.
244. Mohammed EE, Mosad E, Zahran AM, et al. Acridine orange and flow cytometry: Which is better to measure the effect of varicocele on sperm DNA integrity? *Adv Urol.* 2015;2015:814150.
245. Hoshi K, Katayose H, Yanagida K, et al. The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil Steril.* 1996;66:634–9.
246. Skowronek F, Casanova G, Alciaturi J, et al. DNA sperm damage correlates with nuclear ultrastructural sperm defects in teratozoospermic men. *Andrologia.* 2012;44:59–65.
247. Varghese AC, Bragais FM, Mukhopadhyay D, et al. Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics. *Andrologia.* 2009;41: 207–15.
248. Katayose H, Yanagida K, Hashimoto S, et al. Use of diamide-acridine orange fluorescence staining to detect aberrant protamination of human-ejaculated sperm nuclei. *Fertil Steril.* 2003;79:670–6.
249. Lazaros LA, Vartholomatos GA, Hatzi EG, et al. Assessment of sperm chromatin condensation and ploidy status using flow cytometry correlates to fertilization, embryo quality and pregnancy following *in vitro* fertilization. *J Assist Reprod Genet.* 2011;28:885–91.
250. Zini A, Kamal K, Phang D, et al. Biologic variability of sperm DNA denaturation in infertile men. *Urology.* 2001;58:258–61.
251. Muriel L, Meseguer M, Fernandez JL, et al. Value of the sperm chromatin dispersion test in predicting pregnancy outcome in intrauterine insemination: A blind prospective study. *Hum Reprod.* 2006;21:738–44.
252. Muriel L, Garrido N, Fernandez JL, et al. Value of the sperm deoxyribonucleic acid fragmentation level, as measured by the sperm chromatin dispersion test, in the outcome of *in vitro* fertilization and intracytoplasmic sperm injection. *Fertil Steril.* 2006;85:371–83.
253. Muriel L, Goyanes V, Segrelles E, et al. Increased aneuploidy rate in sperm with fragmented DNA as determined by the sperm chromatin dispersion (SCD) test and FISH analysis. *J Androl.* 2007;28:38–49.
254. Fernandez JL, Muriel L, Goyanes V, et al. Halosperm is an easy, available, and cost-effective alternative for determining sperm DNA fragmentation. *Fertil Steril.* 2005;84:860.
255. Gosálvez J, Rodríguez-Predreira M, Mosquera A, et al. Characterisation of a subpopulation of sperm with massive nuclear damage, as recognised with the sperm chromatin dispersion test. *Andrologia.* 2014;46:602–9.
256. Feijó CM, Esteves SC. Diagnostic accuracy of sperm chromatin dispersion test to evaluate sperm deoxyribonucleic acid damage in men with unexplained infertility. *Fertil Steril.* 2014;101: 58–63.e3.
257. Velez de la Calle JF, Muller A, Walschaerts M, et al. Sperm deoxyribonucleic acid fragmentation as assessed by the sperm chromatin dispersion test in assisted reproductive technology programs: Results of a large prospective multicenter study. *Fertil Steril.* 2008;90:1792–9.
258. Cissen M, Wely MV, Scholten I, et al. Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: A systematic review and meta-analysis. *PLoS One.* 2016;11:e0165125.
259. Ribas-Maynou J, Yeste M, Becerra-Tomás N, et al. Clinical implications of sperm DNA damage in IVF and ICSI: Updated systematic review and meta-analysis. *Biol Rev Camb Philos Soc.* 2021;96:1284–1300.
260. Zhang LH, Qiu Y, Wang KH, et al. Measurement of sperm DNA fragmentation using bright-field microscopy: Comparison between sperm chromatin dispersion test and terminal uridine nick-end labeling assay. *Fertil Steril.* 2010;94:1027–32.
261. Meseguer M, Santiso R, Garrido N, et al. Sperm DNA fragmentation levels in testicular sperm samples from azoospermic males as assessed by the sperm chromatin dispersion (SCD) test. *Fertil Steril.* 2009;92:1638–45.
262. Balasuriya A, Speyer B, Serhal P, et al. Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa. *Reprod Biomed Online.* 2011;22:428–36.

263. Santiso R, Tamayo M, Gosálvez J, et al. Simultaneous determination *in situ* of DNA fragmentation and 8-oxoguanine in human sperm. *Fertil Steril.* 2010;93:314–8.
264. Fernández JL, Cajigal D, López-Fernández C, et al. Assessing sperm DNA fragmentation with the sperm chromatin dispersion test. *Methods Mol Biol.* 2011;682:291–301.
265. Ribas-Maynou J, García-Péiró A, Fernández-Encinas A, et al. Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral comet assay. *Andrology.* 2013;1:715–22.
266. Simon L, Carrell DT. Sperm DNA damage measured by comet assay. *Methods Mol Biol.* 2013;927:137–46.
267. Enciso M, Sarasa J, Agarwal A, et al. A two-tailed comet assay for assessing DNA damage in spermatozoa. *Reprod Biomed Online.* 2009;18:609–16.
268. Cortés-Gutiérrez EI, Dávila-Rodríguez MI, Fernández JL, et al. New application of the comet assay: Chromosome-comet assay. *J Histochem Cytochem.* 2011;59:655–60.
269. Hellman B, Vaghef H, Bostrom B. The concepts of tail moment and tail inertia in the single cell gel electrophoresis assay. *Mutat Res.* 1995;336:123–31.
270. Kusakabe H, Tateno H. Shortening of alkaline DNA unwinding time does not interfere with detecting DNA damage to mouse and human spermatozoa in the comet assay. *Asian J Androl.* 2011;13:172–4.
271. Duty SM, Singh NP, Ryan L, et al. Reliability of the comet assay in cryopreserved human sperm. *Hum Reprod.* 2002;17:1274–80.
272. Morris ID, Ilott S, Dixon L, et al. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (comet assay) and its relationship to fertilization and embryo development. *Hum Reprod.* 2002;17:990–8.
273. Simon L, Lutton D, McManus J, et al. Sperm DNA damage measured by the alkaline comet assay as an independent predictor of male infertility and *in vitro* fertilization success. *Fertil Steril.* 2011;95:652–7.
274. Lewis SE, Simon L. Clinical implications of sperm DNA damage. *Hum Fertil (Camb).* 2010;13:201–7.
275. Abu-Hassan D, Koester F, Shoepper B, et al. Comet assay of cumulus cells and spermatozoa DNA status, and the relationship to oocyte fertilization and embryo quality following ICSI. *Reprod Biomed Online.* 2006;12:447–52.
276. Zhang Z, Zhu L, Jiang H, et al. Sperm DNA fragmentation index and pregnancy outcome after IVF or ICSI: A meta-analysis. *J Assist Reprod Genetics.* 2015;32:17–26.
277. Osman A, Alsomait H, Seshadri S, et al. The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: A systematic review and meta-analysis. *Reprod Biomed Online.* 2015;30:120–27.
278. Villani P, Spanò M, Pacchierotti F, et al. Evaluation of a modified comet assay to detect DNA damage in mammalian sperm exposed *in vitro* to different mutagenic compounds. *Reprod Toxicol.* 2010;30:44–9.
279. Benchaib M, Braun V, Lornage J, et al. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod.* 2003;18:1023–8.
280. Enciso M, Johnston SD, Gosálvez J. Differential resistance of mammalian sperm chromatin to oxidative stress as assessed by a two-tailed comet assay. These cells from the genotoxic effects of adverse environments. *Reprod Fertil Dev.* 2011;23:633–7.
281. Sharma R, Agarwal A. Laboratory evaluation of sperm chromatin: TUNEL assay. In: Sperm Chromatin—Biological and Clinical Applications in Male Infertility and Assisted Reproduction. New York, NY: Armand Zini and Ashok Agarwal. Springer Science + Business Media, pp. 201–16, 2011.
282. Mitchell LA, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: Development of an improved methodology. *Int J Androl.* 2011;34:2–13.
283. Carrell DT, Liu L, Peterson CM, et al. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl.* 2003;49:49–55.
284. Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: Correlation with fertilization *in vitro*. *Biol Reprod.* 1997;56:602–7.
285. Cui ZL, Zheng DZ, Liu YH, et al. Diagnostic accuracies of the TUNEL, SCD, and comet based sperm DNA fragmentation assays for male infertility: A meta-analysis study. *Clin Lab.* 2015;61:525–35.
286. Muratori M, Tamburrino L, Tocci V, et al. Small variations in crucial steps of TUNEL assay coupled to flow cytometry greatly affect measures of sperm DNA fragmentation. *J Androl.* 2010;31:336–45.
287. Sharma R, Iovine C, Agarwal A, et al. TUNEL assay-standardized method for testing sperm DNA fragmentation. *Andrologia.* 2021;53:e13738.
288. Spano M, Kolstad AH, Larsen SB, et al. The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies. *Hum Reprod.* 1998;13:2495–505.
289. Nijs M, De Jonge C, Cox A, et al. Correlation between male age, WHO sperm parameters, DNA fragmentation, chromatin packaging and outcome in assisted reproduction technology. *Andrologia.* 2011;43:174–9.
290. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl.* 2002;23:25–43.
291. Bungum M, Bungum L, Giwercman A. Sperm chromatin structure assay (SCSA): A tool in diagnosis and treatment of infertility. *Asian J Androl.* 2011;13:69–75.
292. Castilla JA, Zamora S, Gonzalvo MC, et al. Sperm chromatin structure assay and classical semen parameters: Systematic review. *Reprod Biomed Online.* 2010;20:114–24.
293. Giwercman A, Lindstedt L, Larsson M, et al. Sperm chromatin structure assay as an independent predictor of fertility *in vivo*: A case-control study. *Int J Androl.* 2010;33:e221–7.
294. Miciński P, Pawlicki K, Wielgus E, et al. The sperm chromatin structure assay (SCSA) as prognostic factor in IVF/ICSI program. *Reprod Biol.* 2009;9:65–70.
295. Bach PV, Schlegel PN. Sperm DNA damage and its role in IVF and ICSI. *Basic Clin. Androl.* 2016;26:15.
296. Lewis SE, Agbaje I, Alvarez J. Sperm DNA tests as useful adjuncts to semen analysis. *Syst Biol Reprod Med.* 2008;54:111–25.
297. Evenson DP. Evaluation of sperm chromatin structure and DNA strand breaks is an important part of clinical male fertility assessment. *Transl Androl Urol.* 2017;6:S495–500.
298. Henkel R, Hoogendoijk CF, Bouic PJ, et al. TUNEL assay and SCSA determine different aspects of sperm DNA damage. *Andrologia.* 2010;42:305–13.
299. Vorilhon S, Brugnon F, Kocer A, et al. Accuracy of human sperm DNA oxidation quantification and threshold determination using an 8-OHdG immuno-detection assay. *Hum Reprod.* 2018;33:553–62.
300. Shen H, Ong C. Evaluation of oxidative DNA damage in human sperm and its association with male infertility. *J Androl.* 1999;20:718–23.
301. Aitken RJ, De Iuliis GN, Finnie JM, et al. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: Development of diagnostic criteria. *Hum Reprod.* 2010;25:2415–26.
302. Ishikawa T, Fujioka H, Ishimura T, et al. Increased testicular 8-hydroxy-2'-deoxyguanosine in patients with varicocele. *BJU Int.* 2007;100:863–6.
303. Loft S, Kold-Jensen T, Hjollund NH, et al. Oxidative DNA damage in human sperm influences time to pregnancy. *Hum Reprod.* 2003;18:1265–72.

304. Agarwal A, Varghese AC, Sharma RK. Markers of oxidative stress and sperm chromatin integrity. *Methods Mol Biol.* 2009;590:377–402.
305. Esteves SC, Roque M, Bradley CK, Garrido N. Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: Systematic review and meta-analysis. *Fertil Steril.* 2017;108:456–67.
306. Moskovtsev SI, Jarvi K, Mullen JB, et al. Testicular spermatozoa have statistically significantly lower DNA damage compared with ejaculated spermatozoa in patients with unsuccessful oral antioxidant treatment. *Fertil Steril.* 2010;93:1142–6.
307. Baldini D, Ferri D, Baldini GM, et al. Sperm selection for ICSI: Do we have a winner? *Cells.* 2021;10:3566.
308. Ainsworth C, Nixon B, Aitken RJ. Development of a novel electrophoretic system for the isolation of human spermatozoa. *Hum Reprod.* 2005;20:2261–70.
309. Zini A, San Gabriel M, Baazeem A. Antioxidants and sperm DNA damage: A clinical perspective. *J Assist Reprod Genet.* 2009;26:427–32.
310. Ménézo YJ, Hazout A, Panteix G, et al. Antioxidants to reduce sperm DNA fragmentation: An unexpected adverse effect. *Reprod Biomed Online.* 2007;14:418–21.
311. Agarwal A, Nallella KP, Allamaneni SS, et al. Role of antioxidants in treatment of male infertility: An overview of the literature. *Reprod Biomed Online.* 2004;8:616–27.
312. Rolf C, Cooper TG, Yeung CH, et al. Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: A randomized, placebocontrolled, double-blind study. *Hum Reprod.* 1999;14:1028–33.
313. Said T, Agarwal A, Grunewald S, et al. Selection of nonapoptotic spermatozoa as a new tool for enhancing assisted reproduction outcomes: An *in vitro* model. *Biol Reprod.* 2006;74:530–37.
314. Berkovitz A, Eltes F, Lederman H, et al. How to improve IVF–ICSI outcome by sperm selection. *Reprod Biomed Online.* 2006;12:634–8.
315. Hazout A, Dumont-Hassan M, Junca AM, et al. High-magnification ICSI overcomes paternal effect resistant to conventional ICSI. *Reprod Biomed Online.* 2006;12:19–25.
316. Majumdar G, Majumdar A. A prospective randomized study to evaluate the effect of hyaluronic acid sperm selection on the intracytoplasmic sperm injection outcome of patients with unexplained infertility having normal semen parameters. *J Assist Reprod Genet.* 2013;30:1471–5.
317. Hasanen E, Elqus K, ElTanbouly S, et al. PICSI vs. MACS for abnormal sperm DNA fragmentation ICSI cases: A prospective randomized trial. *J Assist Reprod Genet.* 2020;37:2605–13.
318. Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: A systematic review. *Hum Reprod Update.* 2011;17:719–33.
319. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. *Nature.* 2014;507:181–9.
320. Smith GD, Takayama S. Application of microfluidic technologies to human assisted reproduction. *Mol Hum Reprod.* 2017;23:257–68.
321. Parrella ACD, Keating D, Rosenwaks Z, Palermo GD. A microfluidic devide for selecting the most progressively motile spermatozoa yields a higher rate of euploid embryos. *Fertil Steril.* 2018;110:e342.
322. Khalafalla K, Majzoub A, Elbardisi H, et al. The effect of sperm DNA fragmentation on intracytoplasmic sperm injection outcome. *Andrologia.* 2021;53:e14180.
323. Baskaran S, Agarwal A, Panner Selvam MK, et al. Tracking research trends and hotspots in sperm DNA fragmentation testing for the evaluation of male infertility: A scientometric analysis. *Reprod Biol Endocrinol.* 2019;17:110.

9

OOCYTE RETRIEVAL AND SELECTION

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Introduction

Today, assisted reproductive technologies (ART) refer not only to *in vitro* fertilization (IVF) but to all the approaches tailored to patients' specific conditions. The technology transfer in ART resulted in advanced practices that improved patients' journeys and IVF outcomes.

The reproductive axis is regulated by the pulsatile release of the hypothalamic gonadotropins-releasing hormone (GnRH). It determines the pattern of secretion of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), which then regulate both the endocrine function and gamete maturation in the gonads [1]. Therefore, in IVF, novel protocols regarding ovarian stimulation have been theorized based on the use of gonadotrophins to prompt the growth of antral follicles, to improve the effectiveness and efficacy of IVF treatment in specific patient populations [2].

Controlled ovarian stimulation (COS) entails the administration of exogenous gonadotrophins to stimulate multi-follicular development, a co-treatment with either GnRH agonist or antagonists to suppress pituitary function and prevent premature ovulation, and the trigger of final oocyte maturation 36–38 hours prior to oocyte retrieval [3].

After ovulation triggering, oocyte meiosis (blocked at the prophase of the first meiotic division) is reinitiated, going through germinal vesicle (GV) breakdown and the formation and extrusion of the first polar body (PB1). After entering the second meiotic division, a second arrest occurs at metaphase stage II (MII). The presence of MII spindle together with the PB indicates completion of oocyte maturation, which is required before performing IVF/intracytoplasmic sperm injection (ICSI) procedures. The evaluation of oocyte quality in the laboratory is based on the appearance of the cumulus-oocyte complex (COC) and, after denudation, also on the morphology of the ooplasm and on the aspect of the extra-cytoplasmic structures. However, currently the role of oocyte assessment is controversial; the main aspects evaluated over time to characterize the oocyte developmental competence are summarized in this chapter.

Ovarian stimulation protocols

Prediction of ovarian response

Currently, different stimulants of multi-follicular growth have been suggested, including recombinant, biosimilar or urinary gonadotropins, GnRH analogues (agonists or antagonists), steroid hormones, and other drugs like aromatase inhibitors or growth hormones. The choice of the most suitable COS regimen is based on the prediction of ovarian response so to properly tailor COS [4]. Several predictors have been identified, including maternal age, hormones (FSH and anti-Müllerian hormone [AMH]), morphological parameters (antral follicle count [AFC]), clinical conditions like polycystic ovary syndrome (PCOS), and BMI [5].

Basal serum FSH was adopted as one of the most widespread markers to this end. Yet, its accuracy is limited, and it is only suggested for counselling purposes [6]. The correlation between different groups of patients and basal FSH values is, in fact, statistically not significant, and it does not allow exact classification.

AMH is produced by preantral follicles and small antral follicles of up to 7–8 mm in size. It functions as an inhibitor of FSH-mediated granulosa cell (GC) proliferation, follicular growth, and aromatase activity. AMH level remains stable during menstrual cycles; hence, it is a strong candidate marker for ovarian reserve in women and, in addition, is a strong predictor for the number of oocytes retrieved in patients undergoing IVF treatment.

AFC is conducted through transvaginal ultrasound; it is associated with the ovarian reserve and may predict IVF outcomes. A linear relationship, in fact, exists between AFC and the number of oocytes retrieved [7]. Recently, the combination of AFC and AMH level has been supported as the favourite method to predict the ovarian reserve with a reasonable precision. Single centre receiver operating characteristic curve analyses and meta-analyses showed that both AFC and AMH can identify patients more likely to respond to exogenous gonadotrophins with poor, normal, or hyper-response [7].

Individualized stimulating regimens

Over the past 30 years, particular attention has been paid to the development of simplified ovarian stimulation regimens to identify novel and more convenient approaches to maximize the IVF result. The number of oocytes retrieved is a key factor to improve IVF outcomes [4]. In fact, a large multicentre study reported a significant progressive increase of the cumulative live birth rate (CLBR) with the number of oocytes, thus suggesting that ovarian stimulation may not have a detrimental effect on oocyte/embryo quality [8]. Moreover, it has been assessed that aneuploidy rates do not increase with ovarian response or gonadotropin doses. Also, the number of euploid embryos available for embryo transfer increases as the number of oocytes obtained increases [9]. Different stimulation protocols have been adopted over time to fully exploit the ovarian reserve. In the 1990s, the administration of short-term treatments with GnRH agonist (GnRHa) was reduced in favour of long-term GnRHa stimulation protocols [10]. About 20 years ago, GnRH antagonists were introduced in IVF [11]. These GnRH analogues induce an immediate suppression of the pituitary function, which allows the administration of gonadotropins without pituitary suppression, resulting in shorter and more feasible stimulation protocols [12]. This approach provides similar live birth rates compared with the standard long GnRHa protocols, while minimizing the risk of ovarian hyperstimulation syndrome (OHSS).

Different types of gonadotropins have been adopted to optimize COS. Among them, corifollitropin alfa is a recent therapeutic option developed as an injectable, long-acting FSH agonist. It is a chimeric recombinant molecule composed of

FSH and the carboxy-terminal peptide of human chorionic gonadotropin (hCG). Corifollitropin alfa has a longer half-life compared to recombinant FSH (rFSH) and thus requires less frequent administrations [13]. The results achieved with corifollitropin alfa treatment in terms of clinical pregnancy rate, ongoing pregnancy rate, multiple pregnancy rate, miscarriage rate, ectopic pregnancy rate, and congenital malformation rate (major or minor) was like that obtained with daily treatment of rFSH [14].

Additionally, an unconventional stimulation protocol was introduced that allowed the assessment of the clinical contribution of luteal-phase stimulation (LPS) to follicular-phase stimulation (FPS) in a single ovarian cycle. This protocol is known as DuoStim and it is indicated to poor prognosis patients. It has been reported that a second stimulation in about 15 days resulted in an increased CLBR per ovarian cycle, especially since all embryological, chromosomal, clinical, gestational, and perinatal outcomes are similar between LPS-derived oocytes as FPS-derived ones [15].

Oocyte growth and selection

Oocyte developmental competence is the capacity of a female gamete to reach maturation, be fertilized, and sustain the initial phases of embryonic development until the blastocyst stage [16]. Many factors contribute to the production of a good quality oocyte, and abnormalities in this process lead to infertility and recurrent ART failure. Oocyte quality is not defined only based on its euploid chromosomal constitution but also on all other

aspects that concur to its capacity to reach the blastocyst stage and implant. For instance, limited non-invasive tools are available to permit a proper classification of human oocyte quality prior to fertilization. However, slight deviations from morphological normality should not be considered abnormal. The main factors that could be related to the oocyte quality are summarized in Figure 9.1, and thoroughly described in the following sections.

Perifollicular vascularization evaluation

Oocyte growth, selection, and acquisition of developmental competence is influenced by a dynamic network of blood vessels. This perifollicular vascularization is required to convey oxygen, growth factors, gonadotrophins, and steroid precursors, required to sustain follicle-oocyte maturation [17]. Although several studies demonstrated a strong correlation between a correct vasculature and a healthy follicle development, the cause-effect relation remains poorly understood. Ovarian perifollicular blood flow assessment during IVF using power doppler ultrasound (PDU) has been documented as a good marker of oocyte competence, embryo viability, and implantation potential [18]. Conversely, other studies were not able to confirm the clinical value of the association between perifollicular vascularization and oocyte competence to improve the reproductive outcomes [19, 20]. For these reasons, further prospective randomized studies may clarify the effectiveness of perifollicular vascularization assessment in order to incorporate PDU as a protocol for assessing the gamete/embryo quality.

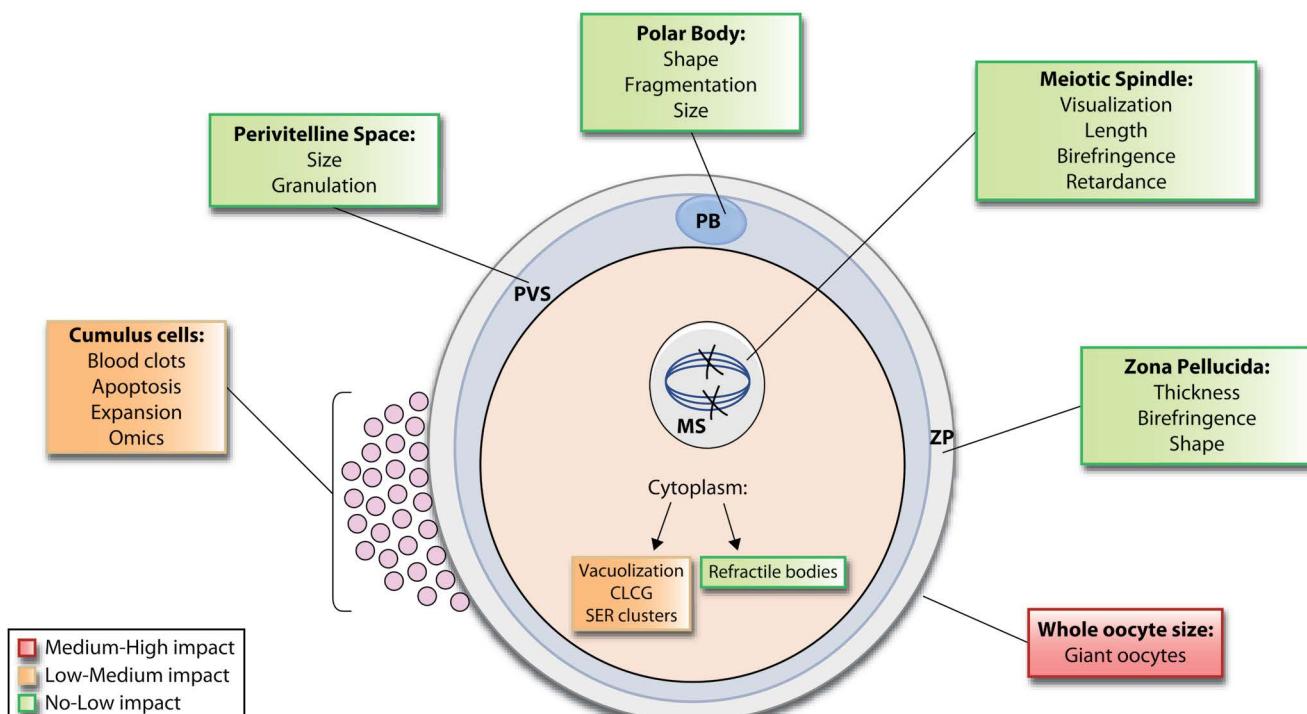


FIGURE 9.1 Extracytoplasmic and intracytoplasmic morphological properties used to assess oocyte competence. The red, orange, and green boxes represent the oocyte dysmorphism with high, medium, and low predictive value of oocyte quality, respectively. Abbreviations: CLCG, centrally located cytoplasmic granulation; SER, smooth endoplasmic reticulum.

Cumulus-oocyte complex evaluation

The mature cumulus-oocyte complex (COC) consists of the secondary oocyte at MII stage, following extrusion of the PB1, surrounded by cumulus cells (CCs). The latter are somatic cells surrounding the oocyte that originate from undifferentiated GCs and promote oocyte growth and maturation. A bi-directional paracrine communication between oocytes and CCs, mediated by the delivery and reception of paracrine factors at the oocyte–GCs interface, regulate the early ovarian follicle development. As CC function is involved in the completion of oocyte meiotic maturation and ovulation, fertilization, and subsequent early embryo development, several authors investigated the morphological features of the COC for the evaluation of oocyte quality.

It was noted that the presence of blood clots in CCs was associated with dense central granulation of oocytes and may adversely affect fertilization, cleavage, and blastulation rates. Blood clots also lead to fewer retrieved oocytes compared with the number of oocytes whose CCs exhibit normal appearance [21]. The adverse effects of blood clots seem to originate from the production of reactive oxygen species (ROS) at high levels from existing blood components [22].

The apoptotic index of CCs was analysed to predict the nuclear maturity of oocytes. The rate of apoptosis in the CCs of immature oocytes at GV or metaphase I (MI) stage is at higher levels than in the CCs from mature oocytes. Moreover, a higher apoptotic rate in CCs of mature oocytes correlates with a lower fertilization rate [23]. Consistently, Faramarzi et al. suggest that oocytes showing a lower apoptotic level independent of cumulus expansion have a better developmental potential [24].

Oocyte developmental potential was also correlated with cumulus expansion. Some studies revealed a positive correlation between cumulus expansion and fertilization rate and blastocyst quality [25, 26]. Although other groups found no difference in the fertilization rate among various COC expansion grades, they found a positive correlation with implantation and pregnancy rates of the relative oocytes [27]. On the contrary, other studies found a negative correlation between the cellular density of the corona radiata and the rate of mature oocytes but not between COC morphology and fertilization, cleavage, and clinical pregnancy rates [21, 28].

Overall, the COC might indicate oocyte quality, and Alpha and ESHRE scientific societies stated that it is reasonable to adopt a binary score to grade compacted CCs (score 0) or expanded CCs and corona radiata (score 1) [29].

Recently, interesting approaches have been developed to better understand oocyte quality based on CCs and GCs RNA/protein content and metabolite production (the so-called-omics technology; e.g. genomic, transcriptomic, proteomic, metabolomic). Thus, it is conceivable that the study of GCs and CCs, which are usually discarded in ICSI treatments, offer a totally non-invasive tool to predict oocyte competence [30, 31].

Current transcriptomics enable qualitative and quantitative characterization of gene expression in cells or tissues. It allows the creation of a list of genes differentially expressed in GCs and CCs surrounding healthy oocytes versus non-viable ones. The data produced to date are still controversial and inconclusive, though, and there is limited consensus on the markers identified [32]. A very recent study from Tiegs and collaborators investigated the presence of differentially expressed genes among CCs of euploid versus embryos affected from trisomy 21, supporting that CCs gene expression may be adopted to identify biomarkers of oocyte

quality [33]. Other studies adopted proteomic and metabolomic approaches to build additional information about the functional status of a certain biological system (i.e. COCs function) [34].

In summary, -omic technologies are still evolving and at present are considered too expensive and time-consuming to be efficiently implemented in the clinical setting. However, soon they might be integrated with morphological analyses to constitute a more efficient platform to assess oocyte/embryo viability [35].

Oocyte nuclear maturity evaluation

Oocyte nuclear maturity is determined by the presence of an extruded PB1 in the perivitelline space (PVS) and by the absence of a GV. The presence of PB1 provides information on the nuclear maturation stage but it does not provide any information on the degree of cytoplasmic maturity. In fact, nuclear and cytoplasmic maturation should be completed in a coordinated manner to ensure optimal conditions for subsequent fertilization. Non-synchronous oocyte maturation is often observed after ovarian stimulation [36]. At the MII stage, the oocyte chromosomes are aligned at the equatorial region of the meiotic spindle (MS). This structure plays a crucial role in the sequence of events leading to the correct completion of meiosis and fertilization and thus is a key determinant of oocyte developmental potential. It has been shown that the MS is highly sensitive to the chemical and physical changes that may occur during oocyte retrieval and handling [37, 38]. Other parameters, such as advanced maternal age [39] and oocyte *in vitro* aging [40], are also associated with the disruption of MS architecture therefore leading to the formation of aneuploid embryos.

The introduction of an orientation-independent polarized light microscopy system allowed the visualization of MS in living oocytes [41]. The absence of a detectable MS and the consequent oocyte developmental impairment may be primarily ascribed to oocyte immaturity. A positive correlation between MS visualization, fertilization rate, and/or embryo development and/or blastocyst progression was described in several studies [42–45]. In contrast, Chamayou et al. reported no correlation between spindle visualization, embryo quality, and clinical pregnancy rates [46]. Interestingly, time-lapse morphokinetic events in conjunction with zona pellucida birefringence (ZPB) and meiotic spindle visualization (MSV) have been assessed for predicting pregnancy outcome by Tabibnejad and collaborators. Implantation, live birth, chemical, and clinical pregnancy rate were reported higher in the transferred embryos deriving from oocytes showing visible spindles [47].

Besides its role in chromosome segregation, the MS is also involved in the extrusion of the PB1. Its position at the very periphery of the cell, attached to the oolemma cortex, dictates the orientation of the cleavage furrow and thus the PB1 extrusion site. However, PB1 has been found frequently dislocated from the MS location after denudation. A moderate degree of PB1/MS deviation does not seem to involve a significant relationship with embryo implantation. However, another possible drawback of PB1 displacement is the potential damage to the MS during ICSI. Consequently, the correct orientation of the oocyte with the MS (and not the PB1) as far as possible from the injection needle allowed ICSI to be safely performed [48].

A possible correlation between MS birefringence, oocyte quality, and embryo development has been suggested [49, 50]. Nevertheless, MS mean retardance, area, and length are not significantly associated with the achievement of a live birth.

Thus, it seems that polarization microscopy cannot be used as a non-invasive marker to predict IVF outcomes [51].

MII oocyte morphological evaluation

An ideal mature human oocyte, based on morphological characteristics, should have a moderately granular cytoplasm; a round and clear ZP, appropriately thick and containing a single, non-fragmented PB1; and a normal PVS [52]. However, many of the oocytes retrieved after ovarian stimulation exhibit one or more morphological abnormalities of the cytoplasm aspect and/or of the extra-cytoplasmic structures [53].

A systematic review of all papers published between 1990 and 2019 aiming at evaluating the predictive value of oocyte morphology suggested that the influence of oocyte dysmorphisms in terms of IVF success is still controversial [54]. The adoption of different criteria for oocyte evaluation may be responsible for the discrepancies across different studies. Alpha and ESHRE scientific societies suggested a common terminology for oocyte morphology assessment to simplify inter-laboratory comparison [29]. Morphological oocyte abnormalities are mainly classified as extra-cytoplasmic features (altered COC morphology, ZP thickness, large PVS, PVS granularity, fragmented or irregular PB1, shape and dimension of the oocyte) or intra-cytoplasmic features (vacuolization, increased cytoplasmic granularity and viscosity, presence of cytoplasmic inclusions, refractile bodies, and smooth endoplasmic reticulum clusters). These morphological properties used to assess oocyte viability are graphically shown in [Figure 9.2](#), along with their predictive value on oocyte quality.

Extra-cytoplasmic abnormalities

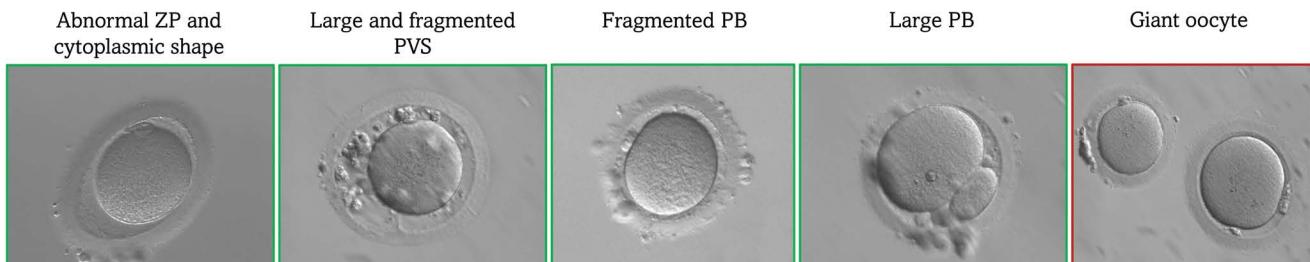
Cumulus-oocyte complex

COC morphological evaluation has been discussed in the “Oocyte–corona–cumulus complex evaluation” paragraph.

Zona pellucida

The ZP is a specialized extracellular matrix layer surrounding the oocyte. Beyond its involvement in oocyte–somatic cell interactions, the ZP also plays an essential role at fertilization, permitting sperm–egg interactions, the acrosome reaction, and an adequate block to polyspermy. Many ZP variants (colour, appearance, thickness, irregularities, composition, and organization) have been described, and abnormal ZP morphology can be observed in 2%–5% of all oocytes [55]. In the past, several studies reported an association between a thicker ZP and decreased fertilization rates, implantation, and pregnancy rates [56, 57]. Oocytes with an oval-shaped ZP have been associated with a high risk of abnormal embryo cleavage and are associated with lower rates of implantation and pregnancy after IVF [58]. However, other analyses, including a randomized controlled trial [59], showed no benefits of ZPs thickness measurements [60–62]. The Istanbul consensus claimed no benefit in measuring zona thickness due to insufficient evidence of any effect on either biological or clinical outcomes. Different colour or thickness of the ZP could be related to patient-specific effects [63]. Additionally, oocytes with heterogeneous ZP (HZP) have a bright vitreous appearance with an irregular outer edge. A study with a limited sample size found that HZP is associated with reduced oocyte maturity, reduced fertilization rates, and lower high-quality embryo rates [64]. Further investigation into the outcome of oocytes with HZP is required. Apparently,

EXTRA-CYTOPLASMATIC ABNORMALITIES



INTRACYTOPLASMATIC ABNORMALITIES

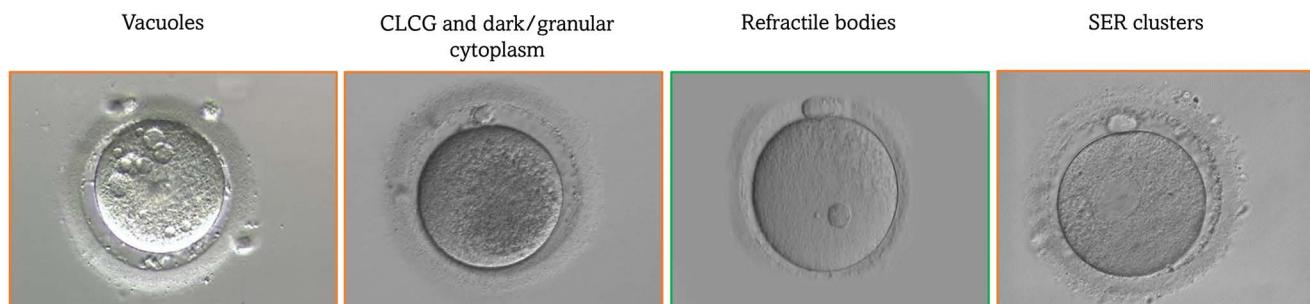


FIGURE 9.2 Examples of extracytoplasmic and intracytoplasmic oocytes abnormalities. The abnormalities with no to low impact on oocytes competence are highlighted in green, in orange if involving low to medium impact, and in red if involving medium to high impact. Abbreviations: PVS, perivitelline space; PB, polar body; CLCG, centrally located cytoplasmic granularity; SER, smooth endoplasmic reticulum.

only drastic morphological alterations (broken or empty ZP) were regarded unsuitable for ICSI.

ZP birefringence, a refractive index obtained from polarization and propagation direction of light, can be analysed to predict the developmental potential of oocytes and early embryos. Oocytes that show a high birefringence of the inner layer of ZP resulted in higher implantation, pregnancy, and live birth rates than that of ones with low birefringence of the inner layer of ZP [44, 50, 65]. The miscarriage rate was also higher in the transferred embryos obtained from oocytes showing low birefringence [44]. On the other hand, another study revealed no differences among high and low birefringence of the inner layer of ZP [47]. In summary, the definition of a potential relationship between ZP characteristics and oocyte competence still requires more studies.

Perivitelline space

PVS represents the acellular compartment in between the plasma membrane of the oocyte and its ZP. It becomes clearly visible in a mature oocyte with the extruded PB located in its most prominent portion. Generally, normal MII oocytes have a small PVS including the single polar body (PB) and no granulated dispersed material [66]. A large PVS seems to reflect an over-maturity of the oocytes at the time of ICSI [67]. Several researchers found that a large PVS may affect oocyte survival [68, 69] and fertilization rate [62] after ICSI. Rienzi and co-authors [62] found that a large PVS is correlated with low fertilization rates and compromised pronuclear morphology, but not with a compromised embryo quality. Moreover, a correlation exists between large PVS and the presence of granulated material in the embryo, but not with implantation and other clinical outcomes [46, 70]. Other different studies failed to report a correlation between the size and shape of the PVS, fertilization rate, and embryo development [60, 71, 72]. However, there was insufficient evidence to support any specific prognosis associated with this observation. In conclusion, an abnormal PVS is insufficient to predict oocyte quality.

First polar body morphology

First polar body (PB1) extrusion indicates completion of the first meiotic division in human oocytes. It has been assumed that PB1 extrusion determines the correct progression of oocyte meiosis in MII and therefore it may mark an important transition into the acquisition of meiotic competence [73]. For this reason, the morphology of the PB could represent a marker of quality. The studies available are distributed between pros and cons, and as a result this assumption is still in debate. Some of these studies showed a correlation between PB1 morphology, such as smooth surface, rough surface, and fragmented with fertilization rate and embryo quality [74, 75] along with implantation and pregnancy rates [76]. According to Rienzi and co-authors [62], abnormal (large or degenerated) PB1 was related to decreased fertilization rates, but no correlation with pronuclear morphology or embryo quality, while fragmentation was not associated with any of these outcomes. Moreover, embryos derived from oocytes with an intact PB1 were more prone to develop into a blastocyst than embryos derived from oocytes with fragmented PB1 [76]. Navarro et al. [77] found a correlation between large PB1 and decreased fertilization, cleavage rates, and compromised embryo quality.

Surprisingly, fragmentation or degeneration of PB1 was found to be related to higher fertilization rates and lower levels of fragmentation of embryos by Fancsovits and co-authors [75], while large PB1 were associated with compromised fertilization and low embryo quality. Conversely, several other studies failed to

demonstrate a relationship between PB1 fragmentation and embryo development [49, 71, 78–80], quality, implantation, or aneuploidies [49, 78, 81]. This discrepancy in the literature seems due to methodological variations in the published studies, sample size, and timing of PB morphology evaluation [82]. Thus, this morphological trait seems a marker of post-ovulatory *in vitro* oocyte aging rather than a proper marker of oocyte quality.

Shape

Good-quality mature human oocytes have a round and clear ZP. Approximately 7% of the oocytes show a deformation of the cytoplasm, which can vary from slightly oval to substantially elongated. An oocyte shape alteration may occur due to mechanical pressure during follicle aspiration or due to the physiological process of folliculogenesis [83]. Oocyte deformation is often associated with ZP abnormalities, and it was thought to be associated with altered embryo cleavage and intercellular blastomeres contacts, in turn affecting compaction and blastocyst formation [84, 85]. Conversely, other studies did not find any correlations between oocyte shape anomalies and fertilization rate, embryo development or embryo quality [86], aneuploidy [87], cryo-survival [62, 79], implantation, and pregnancy [46]. Overall, oocyte shape does not seem to affect IVF outcomes.

Giant oocyte

A giant oocyte is an uncommon abnormality with the incidence around 0.3% among retrieved oocytes [88]. They show about twice the volume of normal oocytes and, presumably, derived from cytoplasmic fusion of two oogonia or nuclear division without cytoplasmic division of an oogonium, therefore originating a tetraploid gamete [89]. It is known that embryos deriving from giant oocytes are at higher risk of digynic triploidy, thus the transfer of embryos derived from them may increase the miscarriage rate [90]. Based on this evidence, giant oocytes should not be used for clinical practice.

Intracytoplasmic abnormalities

Vacuolization

Vacuoles are dynamic cytoplasmic dysmorphisms formed by membrane-bound cytoplasmic inclusions filled with fluid. Vacuoles arise either spontaneously or by fusion of vesicles derived from the smooth endoplasmic reticulum (SER) and/or Golgi apparatus. Intracytoplasmic vacuoles have been associated with severe oocyte degeneration, displacement of the MII spindle from its polar position [91], abnormal cytokinesis pattern [92], compromised embryo development [71], and impaired blastocyst formation [93]. Also, oocytes with vacuoles in the cytoplasm or central granulation exhibited declined cryo-survival and developed into good quality blastocysts less frequently [87]. Rienzi et al. reported a slight but significant decrease in fertilization rates among vacuolated oocytes, but no effect on pronuclear or embryo morphology [62]. The size of the vacuoles might be relevant [94], yet the evidence to date is insufficient to claim that their presence indicates lower oocyte competence.

Cytoplasmic granularity

The presence of centrally located cytoplasmic granulation (CLCG) is considered abnormal and it was originally reported as an indication of cytoplasmic immaturity and lower implantation rate [95]. Merviel et al. found that patients with high ratios (>75%) of CLCG are characterized by lower cleavage, pregnancy, and live birth rates [96]. Rienzi et al. reported that diffuse peripheral

granulation in the MII oocytes resulted in compromised pronuclear morphology [62], as confirmed by several other studies [60, 85]. Ongoing pregnancy rates are seriously compromised in case of CLCG [97–99], perhaps due to allegedly higher embryonic aneuploidies [100]. However, also in this case, the evidence is contrasting, with most of the authors reporting that oocytes with or without CLCG show comparable developmental and reproductive potential [101, 102]. In summary, CLCG is a feature poorly prognostic of oocyte competence.

Dark/granular cytoplasm

The colour variation of the ooplasm is rarely observed and is often described as “dark–granular cytoplasm.” Also, for this feature, the reports are controversial, with some studies reporting an association with a reduced embryo quality [79, 103], and other studies showing no negative correlation [60, 61, 72, 87]. Overall, ooplasm darkness, too, shall be considered a feature with a very limited predictive value.

Cytoplasmic viscosity and refractile bodies

In a limited number of studies, other cytoplasmic changes, such as cytoplasmic viscosity and refractile bodies, were explored. High cytoplasmic viscosity in the oocytes adversely affected fertilization, embryo quality, and blastocyst formation rates [104]. Also, the presence of refractile bodies derived from lipofuscin inclusions correlated with the decreased fertilization rate and defective blastocyst formation [105]. However, other researchers reported that refractile bodies and cytoplasmic viscosity in oocytes do not impact on embryological (fertilization and embryo quality) and clinical (implantation and pregnancy) outcomes [60, 72].

Smooth endoplasmic reticulum aggregates

SER aggregates (SER-a) look like fat disks in the oocyte cytoplasm corresponding to large tubular SER clusters surrounded by mitochondria. SER is a structure dedicated to calcium storage and release, necessary for oocyte activation at fertilization. Moreover, it plays a crucial role in energy accumulation, synthesis of proteins, lipids, and nuclear membrane throughout early embryo development [106]. SER-a were associated with lower oocyte maturation and embryo quality; lower fertilization, implantation, and pregnancy rates; and increased miscarriage rates when compared with control oocytes [84, 106–108]. SER-a presence has been associated with cytoskeleton alterations including increased spindle length and cortical actin disorganization [109, 110]. The potential association between SER-a and certain imprinting disorders [106, 111–113] led embryologists to discard these oocytes for several years, as initially suggested also by the main international scientific societies in the field of ART. However, more recent evidence suggests that embryos derived from oocytes with SER-a undergo a normal development, resulting in healthy newborns [114]. Considering all these controversial data and the lack of a real link between oocytes with SER-a and a higher prevalence of aneuploidies and/or malformations, discarding them cannot be justified clinically. Indeed, they can be valuable especially for poor prognosis patients. It is clearly advisable to monitor the embryos and babies born deriving from oocytes with SER-a in their cytoplasm.

Cumulative effect of multiple abnormalities

Regarding the cumulative effect of multiple morphological features, Xia [115] showed that oocyte grading based on PB1 morphology, size of PVS, and cytoplasmic inclusions was correlated

with its developmental potential after ICSI. In the study of Chamayou et al. [46], the cumulative effect of morphological features, including cytoplasmic texture, inclusions, vacuoles, refractile bodies, and central granulation, was found related to impaired embryo quality but did not influence pregnancy rates. A completely different conclusion has been obtained by Serhal and co-authors [95], who found that similar features did not influence *in vitro* developmental parameters, but implantation and pregnancy rates were lower when embryos were derived from oocytes with cytoplasmic abnormalities.

Conclusions

Full oocyte maturation requires nuclear and cytoplasmic changes that must be completed in a timely manner to ensure optimal cellular conditions. An altered nuclear or cytoplasmic maturation or their asynchrony may compromise oocyte quality, resulting in various oocyte dysmorphisms. Nevertheless, most of the oocytes retrieved after oocyte stimulation exhibit one or more of these abnormal morphologic characteristics. To date, all these MII oocytes morphological parameters resulted in a very limited predictive value because of contradicting results, therefore their clinical use to foresee oocyte competence cannot be supported. The only morphological anomaly with clear clinical consequences is oocyte size (giant oocytes); all the remaining abnormalities are ascribable to phenotypic variance. Assessing chromosomal abnormalities at the blastocyst stage remains the most powerful and effective approach to predict embryo competence. Further relevant information could be obtained from blastocyst morphological assessment and perhaps “-omics technologies” in the future, while the predictive power of oocyte morphology with respect to embryo competence remains very limited.

References

1. le Nestour E, Marraoui J, Lahlou N, Roger M, de Ziegler D, Bouchard P. Role of estradiol in the rise in follicle-stimulating hormone levels during the luteal-follicular transition. *J Clin Endocrinol Metab.* 1993;77(2):439–42.
2. Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, Kolibianakis E, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI(dagger). *Hum Reprod Open.* 2020;2020(2):hoaa009.
3. Gallos ID, Eapen A, Price MJ, Sunkara SK, Macklon NS, Bhattacharya S, et al. Controlled ovarian stimulation protocols for assisted reproduction: A network meta-analysis. *The Cochrane Data Syst Rev.* 2017;2017(3):CD012586. doi: [10.1002/14651858](https://doi.org/10.1002/14651858.CD012586).
4. Sunkara SK, Rittenberg V, Raine-Fenning N, Bhattacharya S, Zamora J, Coomarasamy A. Association between the number of eggs and live birth in IVF treatment: An Analysis of 400 135 treatment cycles. *Hum Reprod.* 2011;26(7):1768–74.
5. Ubaldi F, Vaiarelli A, D'Anna R, Rienzi L. Management of poor responders in IVF: Is there anything new? *Biomed Res Int.* 2014;2014:352098.
6. Broekmans F, Kwee J, Hendriks D, Mol B, Lambalk C. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update.* 2006;12(6):685–718.
7. Tsakos E, Tolikas A, Daniilidis A, Asimakopoulos B. Predictive value of anti-Müllerian hormone, follicle-stimulating hormone and antral follicle count on the outcome of ovarian stimulation in women following GnRH-antagonist protocol for IVF/ET. *Arch Gynecol Obstetr.* 2014;290(6):1249–53.
8. Polyzos NP, Drakopoulos P, Parra J, Pellicer A, Santos-Ribeiro S, Tournaye H, et al. Cumulative live birth rates according to the number of oocytes retrieved after the first ovarian stimulation

- for in vitro fertilization/intracytoplasmic sperm injection: A multicenter multinational analysis including approximately 15,000 women. *Fertil Steril.* 2018;110(4):661–70 e1.
9. Labarta E, Bosch E, Mercader A, Alama P, Mateu E, Pellicer A. A higher ovarian response after stimulation for IVF is related to a higher number of euploid embryos. *Biomed Res Int.* 2017;2017: 5637923.
 10. Fauser BC, Devroey P, Yen SS, Gosden R, Crowley WF Jr, Baird DT, et al. Minimal ovarian stimulation for IVF: Appraisal of potential benefits and drawbacks. *Hum Reprod.* 1999;14(11):2681–6.
 11. Albano C, Felberbaum R, Smits J, Riethmuller-Winzen H, Engel J, Diedrich K, et al. Ovarian stimulation with HMG: Results of a prospective randomized phase III European study comparing the luteinizing hormone-releasing hormone (LHRH)-antagonist cetrorelix and the LHRH-agonist buserelin. *Hum Reprod.* 2000;15(3):526–31.
 12. Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. *J Clin Endocrinol Metab.* 2003;88(1):166–73.
 13. Fauser BC, Alper MM, Ledger W, Schoolcraft WB, Zandvliet A, Mannaerts BM, et al. Pharmacokinetics and follicular dynamics of corifollitropin alfa versus recombinant FSH during ovarian stimulation for IVF. *Reprod Biomed Online.* 2010;21(5):593–601.
 14. Pouwer AW, Farquhar C, Kremer JA. Long-acting FSH versus daily FSH for women undergoing assisted reproduction. *Cochrane Data Syst Rev.* 2015(7):CD009577.
 15. Vaiarelli A, Cimadomo D, Alviggi E, Sansone A, Trabucco E, Dusi L, et al. The euploid blastocysts obtained after luteal phase stimulation show the same clinical, obstetric and perinatal outcomes as follicular phase stimulation-derived ones: A multicenter study. *Hum Reprod.* 2020;35(11):2598–608.
 16. Conti M, Franciosi F. Acquisition of oocyte competence to develop as an embryo: Integrated nuclear and cytoplasmic events. *Hum Reprod Update.* 2018;24(3):245–66.
 17. Rizov M, Andreeva P, Dimova I. Molecular regulation and role of angiogenesis in reproduction. *Taiwan J Obstet Gynecol.* 2017; 56(2):127–32.
 18. Naredi N, Singh SK, Sharma R. Does perifollicular vascularity on the day of oocyte retrieval affect pregnancy outcome in an in vitro fertilization cycle? *J Hum Reprod Sci.* 2017;10(4):281–7.
 19. Kan A, Ng EHY, Yeung WSB, Ho PC. Perifollicular vascularity in poor ovarian responders during IVF. *Hum Reprod.* 2006;21(6): 1539–44.
 20. Ng EHY, Tang OS, Chan CCW, Ho PC. Ovarian stromal vascularity is not predictive of ovarian response and pregnancy. *Reprod Biomed Online.* 2006;12(1):43–9.
 21. Ebner T, Moser M, Shebl O, Sommergruber M, Yaman C, Tews G. Blood clots in the cumulus-oocyte complex predict poor oocyte quality and post-fertilization development. *Reprod Biomed Online.* 2008;16(6):801–7.
 22. Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A, et al. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. *Int J Fertil Womens Med.* 2000;45(5):314–20.
 23. Host E, Gabrielsen A, Lindenberg S, Smidt-Jensen S. Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage, and cleavage of the corresponding oocyte after intracytoplasmic sperm injection. *Fertil Steril.* 2002;77(3): 511–5.
 24. Faramarzi A, Khalili MA, Omidi M. Morphometric analysis of human oocytes using time lapse: Does it predict embryo developmental outcomes? *Hum Fertil (Camb).* 2019;22(3):171–6.
 25. Lin YC, Chang SY, Lan KC, Huang HW, Chang CY, Tsai MY, et al. Human oocyte maturity in vivo determines the outcome of blastocyst development in vitro. *J Assist Reprod Genet.* 2003;20(12):506–12.
 26. Ng ST, Chang TH, Wu TC. Prediction of the rates of fertilization, cleavage, and pregnancy success by cumulus-coronal morphology in an in vitro fertilization program. *Fertil Steril.* 1999;72(3): 412–7.
 27. Dal Canto M, Brambillasca F, Mignini Renzini M, Coticchio G, Merola M, Lain M, et al. Cumulus cell-oocyte complexes retrieved from antral follicles in IVM cycles: Relationship between COCs morphology, gonadotropin priming and clinical outcome. *J Assist Reprod Genet.* 2012;29(6):513–9.
 28. Rattanachaiyanont M, Leader A, Leveille MC. Lack of correlation between oocyte-corona-cumulus complex morphology and nuclear maturity of oocytes collected in stimulated cycles for intracytoplasmic sperm injection. *Fertil Steril.* 1999;71(5): 937–40.
 29. Balaban B, Thornhill A. Alpha–Scientists in reproductive medicine. *Reprod Biomed Online.* 2011;23:68.
 30. Huang Z, Wells D. The human oocyte and cumulus cells relationship: New insights from the cumulus cell transcriptome. *Mol Hum Reprod.* 2010;16(10):715–25.
 31. Royere D, Feuerstein P, Cadoret V, Puard V, Uzbekova S, Dalbies-Tran R, et al. [Non invasive assessment of embryo quality: Proteomics, metabolomics and oocyte-cumulus dialogue]. *Gynecol Obstet Fertil.* 2009;37(11-12):917–20.
 32. Adriaenssens T, Wathlet S, Segers I, Verheyen G, De Vos A, Van der Elst J, et al. Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics. *Hum Reprod.* 2010;25(5):1259–70.
 33. Tiegs AW, Titus S, Mehta S, Garcia-Milian R, Seli E, Scott RT Jr. Cumulus cells of euploid versus whole chromosome 21 aneuploid embryos reveal differentially expressed genes. *Reprod Biomed Online.* 2021;43(4):614–26.
 34. Revelli A, Piane LD, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: From single biochemical markers to metabolomics. *Reprod Biol Endocrinol.* 2009;7(1): 1–13.
 35. Pandit S, Sharma R. Non invasive assessment of human oocytes and embryos in assisted reproduction: Review on present practices and future trends. *Med J Armed Forces India.* 2022;78(1): 7–16.
 36. Ubaldi F, Rienzi L. Micromanipulation techniques in human infertility: PZD, SUZI, ICSI, MESA, PESA, FNA and TESE. *Biotechnol Hum Reprod.* 2003;315–36.
 37. Eichenlaub-Ritter U, Shen Y, Tinneberg HR. Manipulation of the oocyte: Possible damage to the spindle apparatus. *Reprod Biomed Online.* 2002;5(2):117–24.
 38. Wang WH, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. *Hum Reprod.* 2001;16(11):2374–8.
 39. Volarcik K, Sheean L, Goldfarb J, Woods L, Abdul-Karim FW, Hunt P. The meiotic competence of in-vitro matured human oocytes is influenced by donor age: Evidence that folliculogenesis is compromised in the reproductively aged ovary. *Hum Reprod.* 1998;13(1):154–60.
 40. Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R. Spindles, mitochondria and redox potential in ageing oocytes. *Reprod Biomed Online.* 2004;8(1):45–58.
 41. Liu L, Trimarchi JR, Oldenbourg R, Keefe DL. Increased birefringence in the meiotic spindle provides a new marker for the onset of activation in living oocytes. *Biol Reprod.* 2000;63(1): 251–8.
 42. Rienzi L, Ubaldi F, Martinez F, Jacobelli M, Minasi MG, Ferrero S, et al. Relationship between meiotic spindle location with regard to the polar body position and oocyte developmental potential after ICSI. *Hum Reprod.* 2003;18(6):1289–93.
 43. Cohen Y, Malcov M, Schwartz T, Mey-Raz N, Carmon A, Cohen T, et al. Spindle imaging: A new marker for optimal timing of ICSI? *Hum Reprod.* 2004;19(3):649–54.

44. Madaschi C, Aoki T, de Almeida Ferreira Braga DP, de Cassia Savio Figueira R, Semiao Francisco L, Iaconelli A Jr., et al. Zona pellucida birefringence score and meiotic spindle visualization in relation to embryo development and ICSI outcomes. *Reprod Biomed Online.* 2009;18(5):681–6.
45. Gonzalez-Ortega C, Cancino-Villarreal P, Alonso-Torres VE, Martinez-Robles I, Perez-Pena E, Gutierrez-Gutierrez AM. [Polarized light microscopy for evaluation of oocytes as a prognostic factor in the evolution of a cycle in assisted reproduction]. *Ginecol Obstet Mex.* 2016;84(4):217–27.
46. Chamayou S, Ragolia C, Alecci C, Storaci G, Maglia E, Russo E, et al. Meiotic spindle presence and oocyte morphology do not predict clinical ICSI outcomes: A study of 967 transferred embryos. *Reprod Biomed Online.* 2006;13(5):661–7.
47. Tabibnejad N, Soleimani M, Aflatoonian A. Zona pellucida birefringence and meiotic spindle visualization are not related to the time-lapse detected embryo morphokinetics in women with polycystic ovarian syndrome. *Eur J Obstet Gynecol Reprod Biol.* 2018;230:96–102.
48. Rienzi L, Ubaldi F, Iacobelli M, Minasi MG, Romano S, Greco E. Meiotic spindle visualization in living human oocytes. *Reprod Biomed Online.* 2005;10(2):192–8.
49. De Santis L, Cino I, Rabbolotti E, Calzi F, Persico P, Borini A, et al. Polar body morphology and spindle imaging as predictors of oocyte quality. *Reprod Biomed Online.* 2005;11(1):36–42.
50. Rama Raju GA, Prakash GJ, Krishna KM, Madan K. Meiotic spindle and zona pellucida characteristics as predictors of embryonic development: A preliminary study using PolScope imaging. *Reprod Biomed Online.* 2007;14(2):166–74.
51. Swiatecka J, Bielawski T, Anchim T, Lesniewska M, Milewski R, Wolczynski S. Oocyte zona pellucida and meiotic spindle birefringence as a biomarker of pregnancy rate outcome in IVF-ICSI treatment. *Ginekol Pol.* 2014;85(4):264–71.
52. Swain JE, Pool TB. ART failure: Oocyte contributions to unsuccessful fertilization. *Hum Reprod Update.* 2008;14(5):431–46.
53. Ozturk S. Selection of competent oocytes by morphological criteria for assisted reproductive technologies. *Mol Reprod Dev.* 2020;87(10):1021–36.
54. Bartolacci A, Intra G, Coticchio G, dell'Aquila M, Patria G, Borini A. Does morphological assessment predict oocyte developmental competence? A systematic review and proposed score. *J Assist Reprod Genet.* 2022;39(1):3–17.
55. Rienzi L, Vajta G, Ubaldi F. Predictive value of oocyte morphology in human IVF: A systematic review of the literature. *Hum Reprod Update.* 2011;17(1):34–45.
56. Bertrand E, Van den Bergh M, Englert Y. Does zona pellucida thickness influence the fertilization rate? *Hum Reprod.* 1995;10(5): 1189–93.
57. Gabrielsen A, Bhatnager PR, Petersen K, Lindenberg S. Influence of zona pellucida thickness of human embryos on clinical pregnancy outcome following in vitro fertilization treatment. *J Assist Reprod Genet.* 2000;17(6):323–8.
58. Sauerbrun-Cutler MT, Vega M, Breborowicz A, Gonzales E, Stein D, Lederman M, et al. Oocyte zona pellucida dysmorphology is associated with diminished in-vitro fertilization success. *J Ovarian Res.* 2015;8:5.
59. Gabrielsen A, Lindenberg S, Petersen K. The impact of the zona pellucida thickness variation of human embryos on pregnancy outcome in relation to suboptimal embryo development. A prospective randomized controlled study. *Hum Reprod.* 2001;16(10): 2166–70.
60. Balaban B, Urman B, Sertac A, Alatas C, Aksoy S, Mercan R. Oocyte morphology does not affect fertilization rate, embryo quality and implantation rate after intracytoplasmic sperm injection. *Hum Reprod.* 1998;13(12):3431–3.
61. Esfandiari N, Burjaq H, Gotlieb L, Casper RF. Brown oocytes: Implications for assisted reproductive technology. *Fertil Steril.* 2006;86(5):1522–5.
62. Rienzi L, Ubaldi FM, Iacobelli M, Minasi MG, Romano S, Ferrero S, et al. Significance of metaphase II human oocyte morphology on ICSI outcome. *Fertil Steril.* 2008;90(5):1692–700.
63. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Hum Reprod.* 2011;26(6):1270–83.
64. Li M, Ma SY, Yang HJ, Wu KL, Zhong WX, Yu GL, et al. Pregnancy with oocytes characterized by narrow perivitelline space and heterogeneous zona pellucida: Is intracytoplasmic sperm injection necessary? *J Assist Reprod Genet.* 2014;31(3):285–94.
65. Montag M, Schimming T, Koster M, Zhou C, Dorn C, Rosing B, et al. Oocyte zona birefringence intensity is associated with embryonic implantation potential in ICSI cycles. *Reprod Biomed Online.* 2008;16(2):239–44.
66. Ubaldi F, Rienzi L. Morphological selection of gametes. *Placenta.* 2008;29(Suppl B):115–20.
67. Mikkelsen AL, Lindenberg S. Morphology of in-vitro matured oocytes: Impact on fertility potential and embryo quality. *Hum Reprod.* 2001;16(8):1714–8.
68. Ebner T, Yaman C, Moser M, Sommergruber M, Jesacher K, Tews G. A prospective study on oocyte survival rate after ICSI: Influence of injection technique and morphological features. *J Assist Reprod Genet.* 2001;18(12):623–8.
69. Plachot M, Selva J, Wolf JP, Bastit P, de Mouzon J. Consequences of oocyte dysmorphism on the fertilization rate and embryo development after intracytoplasmic sperm injection. A prospective multi-center study. *Ginekol Obstet Fertil.* 2002;30(10):772–9.
70. Hassan-Ali H, Hisham-Saleh A, El-Gezeiry D, Baghdady I, Ismaeil I, Mandelbaum J. Perivitelline space granularity: A sign of human menopausal gonadotrophin overdose in intracytoplasmic sperm injection. *Hum Reprod.* 1998;13(12):3425–30.
71. Balaban B, Urman B. Effect of oocyte morphology on embryo development and implantation. *Reprod Biomed Online.* 2006;12(5): 608–15.
72. De Sutter P, Dozortsev D, Qian C, Dhont M. Oocyte morphology does not correlate with fertilization rate and embryo quality after intracytoplasmic sperm injection. *Hum Reprod.* 1996;11(3): 595–7.
73. Younis JS, Radin O, Izhaki I, Ben-Ami M. Does first polar body morphology predict oocyte performance during ICSI treatment? *J Assist Reprod Genet.* 2009;26(11–12):561–7.
74. Ebner T, Yaman C, Moser M, Sommergruber M, Feichtinger O, Tews G. Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection. *Hum Reprod.* 2000;15(2):427–30.
75. Fancsovits P, Tothne ZG, Murber A, Takacs FZ, Papp Z, Urbancsek J. Correlation between first polar body morphology and further embryo development. *Acta Biol Hung.* 2006;57(3): 331–8.
76. Ebner T, Moser M, Sommergruber M, Yaman C, Pfleger U, Tews G. First polar body morphology and blastocyst formation rate in ICSI patients. *Hum Reprod.* 2002;17(9):2415–8.
77. Navarro PA, de Araujo MM, de Araujo CM, Rocha M, dos Reis R, Martins W. Relationship between first polar body morphology before intracytoplasmic sperm injection and fertilization rate, cleavage rate, and embryo quality. *Int J Gynaecol Obstet.* 2009; 104(3):226–9.
78. Verlinsky Y, Lerner S, Illkevitch N, Kuznetsov V, Kuznetsov I, Cieslak J, et al. Is there any predictive value of first polar body morphology for embryo genotype or developmental potential? *Reprod Biomed Online.* 2003;7(3):336–41.
79. Ten J, Mendiola J, Vioque J, de Juan J, Bernabeu R. Donor oocyte dysmorphisms and their influence on fertilization and embryo quality. *Reprod Biomed Online.* 2007;14(1):40–8.
80. Zhou W, Fu L, Sha W, Chu D, Li Y. Relationship of polar bodies morphology to embryo quality and pregnancy outcome. *Zygote.* 2016;24(3):401–7.

81. Ciotti PM, Notarangelo L, Morselli-Labate AM, Felletti V, Porcu E, Venturoli S. First polar body morphology before ICSI is not related to embryo quality or pregnancy rate. *Hum Reprod.* 2004;19(10):2334–9.
82. Halvaei I, Khalili MA, Soleimani M, Razi MH. Evaluating the role of first polar body morphology on rates of fertilization and embryo development in ICSI cycles. *Int J Fertil Steril.* 2011;5(2):110–5.
83. Shen Y, Stalf T, Mehnert C, Eichenlaub-Ritter U, Tinneberg HR. High magnitude of light retardation by the zona pellucida is associated with conception cycles. *Hum Reprod.* 2005;20(6):1596–606.
84. Braga DP, Setti AS, Figueira Rde C, Machado RB, Iaconelli A Jr., Borges E Jr. Influence of oocyte dysmorphisms on blastocyst formation and quality. *Fertil Steril.* 2013;100(3):748–54.
85. Ebner T, Shebl O, Moser M, Sommergruber M, Tews G. Developmental fate of ovoid oocytes. *Hum Reprod.* 2008;23(1):62–6.
86. Balaban B, Urman B, Sertac A, Alatas C, Aksoy S, Mercan R, et al. In-vitro culture of spermatozoa induces motility and increases implantation and pregnancy rates after testicular sperm extraction and intracytoplasmic sperm injection. *Hum Reprod.* 1999;14(11):2808–11.
87. Balaban B, Ata B, Isiklar A, Yakin K, Urman B. Severe cytoplasmic abnormalities of the oocyte decrease cryosurvival and subsequent embryonic development of cryopreserved embryos. *Hum Reprod.* 2008;23(8):1778–85.
88. Rosenbusch B. The potential significance of binovular follicles and binucleate giant oocytes for the development of genetic abnormalities. *J Genet.* 2012;91(3):397–404.
89. Austin CR. Anomalies of fertilization leading to triploidy. *J Cell Comp Physiol.* 1960;56(Suppl 1):1–15.
90. Balakier H, Bouman D, Sojecki A, Librach C, Squire JA. Morphological and cytogenetic analysis of human giant oocytes and giant embryos. *Hum Reprod.* 2002;17(9):2394–401.
91. Nayudu PL, Lopata A, Jones GM, Gook DA, Bourne HM, Sheather SJ, et al. An analysis of human Oocytes and follicles from stimulated cycles: Oocyte morphology and associated follicular fluid characteristics. *Hum Reprod.* 1989;4(5):558–67.
92. Van Blerkom J. Occurrence and developmental consequences of aberrant cellular organization in meiotically mature human oocytes after exogenous ovarian hyperstimulation. *J Electron Microsc Tech.* 1990;16(4):324–46.
93. Ebner T, Moser M, Sommergruber M, Gaiswinkler U, Shebl O, Jesacher K, et al. Occurrence and developmental consequences of vacuoles throughout preimplantation development. *Fertil Steril.* 2005;83(6):1635–40.
94. Wallbutton S, Kasraie J. Vacuolated oocytes: Fertilization and embryonic arrest following intra-cytoplasmic sperm injection in a patient exhibiting persistent oocyte macro vacuolization—case report. *J Assist Reprod Genet.* 2010;27(4):183–8.
95. Serhal PF, Ranieri DM, Kinis A, Marchant S, Davies M, Khadum IM. Oocyte morphology predicts outcome of intracytoplasmic sperm injection. *Hum Reprod.* 1997;12(6):1267–70.
96. Merviel P, Cabry R, Chardon K, Haraux E, Scheffler F, Mansouri NB, et al. Impact of oocytes with CLCG on ICSI outcomes and their potential relation to pesticide exposure. *J Ovarian Res.* 2017;10(1):42.
97. Alikani M, Palermo G, Adler A, Bertoli M, Blake M, Cohen J. Intracytoplasmic sperm injection in dysmorphic human oocytes. *Zygote.* 1995;3(4):283–8.
98. Kahraman S, Yakin K, Donmez E, Samli H, Bahce M, Cengiz G, et al. Relationship between granular cytoplasm of oocytes and pregnancy outcome following intracytoplasmic sperm injection. *Hum Reprod.* 2000;15(11):2390–3.
99. Tulay P, Arslan H, Buran A, Koprulu Y. Assessment of successful pregnancy using granular oocytes in ICSI treatments. *Zygote.* 2019;27(2):97–100.
100. Schmutzler AG, Acar-Perk B, Weimer J, Salmassi A, Sievers K, Tobler M, et al. Oocyte morphology on day 0 correlates with aneuploidy as detected by polar body biopsy and FISH. *Arch Gynecol Obstet.* 2014;289(2):445–50.
101. Setti AS, Figueira RC, Braga DP, Colturato SS, Iaconelli A Jr., Borges E Jr. Relationship between oocyte abnormal morphology and intracytoplasmic sperm injection outcomes: A meta-analysis. *Eur J Obstet Gynecol Reprod Biol.* 2011;159(2):364–70.
102. Yi XF, Xi HL, Zhang SL, Yang J. Relationship between the positions of cytoplasmic granulation and the oocytes developmental potential in human. *Sci Rep.* 2019;9(1):7215.
103. Loutradis D, Drakakis P, Kallianidis K, Milingos S, Dendrinou S, Michalas S. Oocyte morphology correlates with embryo quality and pregnancy rate after intracytoplasmic sperm injection. *Fertil Steril.* 1999;72(2):240–4.
104. Ebner T, Moser M, Sommergruber M, Puchner M, Wiesinger R, Tews G. Developmental competence of oocytes showing increased cytoplasmic viscosity. *Hum Reprod.* 2003;18(6):1294–8.
105. Otsuki J, Nagai Y, Chiba K. Lipofuscin bodies in human oocytes as an indicator of oocyte quality. *J Assist Reprod Genet.* 2007;24(7):263–70.
106. Sa R, Cunha M, Silva J, Luis A, Oliveira C, Teixeira da Silva J, et al. Ultrastructure of tubular smooth endoplasmic reticulum aggregates in human metaphase II oocytes and clinical implications. *Fertil Steril.* 2011;96(1):143–9 e7.
107. Ferreux L, Sallem A, Chargui A, Gille AS, Bourdon M, Maignien C, et al. Is it time to reconsider how to manage oocytes affected by smooth endoplasmic reticulum aggregates? *Hum Reprod.* 2019;34(4):591–600.
108. Setti AS, Figueira RC, de Almeida Ferreira Braga DP, Azevedo MC, Iaconelli A Jr., Borges E Jr. Oocytes with smooth endoplasmic reticulum clusters originate blastocysts with impaired implantation potential. *Fertil Steril.* 2016;106(7):1718–24.
109. Dal Canto M, Guglielmo MC, Mignini Renzini M, Fadini R, Moutir C, Merola M, et al. Dysmorphic patterns are associated with cytoskeletal alterations in human oocytes. *Hum Reprod.* 2017;32(4):750–7.
110. Stigliani S, Moretti S, Casciano I, Canepa P, Remorgida V, Anserini P, et al. Presence of aggregates of smooth endoplasmic reticulum in MII oocytes affects oocyte competence: Molecular-based evidence. *Mol Hum Reprod.* 2018;24(6):310–7.
111. Otsuki J, Okada A, Morimoto K, Nagai Y, Kubo H. The relationship between pregnancy outcome and smooth endoplasmic reticulum clusters in MII human oocytes. *Hum Reprod.* 2004;19(7):1591–7.
112. Akarsu C, Caglar G, Vicdan K, Sozen E, Biberoglu K. Smooth endoplasmic reticulum aggregations in all retrieved oocytes causing recurrent multiple anomalies: Case report. *Fertil Steril.* 2009;92(4):1496.e1–e3.
113. Ebner T, Moser M, Shebl O, Sommergruber M, Tews G. Prognosis of oocytes showing aggregation of smooth endoplasmic reticulum. *Reprod Biomed Online.* 2008;16(1):113–8.
114. Shaw-Jackson C, Van Beirs N, Thomas AL, Rozenberg S, Autin C. Can healthy babies originate from oocytes with smooth endoplasmic reticulum aggregates? A systematic mini-review. *Hum Reprod.* 2014;29(7):1380–6.
115. Xia P. Intracytoplasmic sperm injection: Correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality. *Hum Reprod.* 1997;12(8):1750–5.

10

PREPARATION AND EVALUATION OF OOCYTES FOR INTRACYTOPLASMIC SPERM INJECTION

Irit Granot and Nava Dekel

Introduction

Resumption of meiosis in the oocyte is an essential prelude to successful fertilization. The meiotic division of the mammalian oocyte is initiated during fetal life, proceeds up to the diplotene stage of the first prophase, and arrests at birth. Meiotic arrest persists throughout childhood until the onset of puberty. In a sexually mature female, at each cycle one or more oocytes, according to the species, re-initiate the meiotic division. The chromatin in the meiotically arrested oocytes is encapsulated by a nuclear structure known as the germinal vesicle (GV; [Figure 10.1a](#)). The GV in oocytes resuming meiosis disappears ([Figure 10.1b](#)), the condensed chromosomes align on the newly formed meiotic spindle, and the pairs of homologous chromosomes segregate between the oocyte and the first polar body ([Figure 10.1c](#)). Emission of the first polar body, which represents the completion of the first round of meiotic division, is immediately followed by the formation of the second meiotic spindle with the remaining set of homologous chromosomes aligned on its equatorial plate. The whole series of events, initiated by GV breakdown (GVB) and completed at the metaphase of the second round of meiosis (MII), leads to the production of a mature fertilizable oocyte, also known as an egg. The egg is arrested at MII and will complete the meiotic division only after the penetration of the spermatozoon [[1](#)]. The physiological stimulus for re-initiation of meiosis is provided by the pre-ovulatory surge of luteinizing hormone (LH) [[2](#)]. Once oocyte maturation is completed, LH further induces ovulation, during which the follicle releases the mature oocyte that is picked up by the infundibular fimbria of the oviduct.

The egg released from the ovarian follicle is accompanied by the cumulus cells. Prior to ovulation, in concomitance with oocyte maturation, the cumulus undergoes characteristic transformations that are also stimulated by LH. In response to this gonadotropin, the cumulus cells produce specific glycosaminoglycans, the secretion of which results in cumulus mucification and its expansion. The major component of the extracellular matrix secreted by the cumulus cells is hyaluronic acid [[3–7](#)]. The mucified cumulus mass that encapsulates the ovulated egg is penetrated by the spermatozoon that uses enzymes localized on its surface membrane to accomplish this mission. Sperm membrane protein PH-20, which is present on the plasma membrane of sperm cells of many species, such as guinea pigs, mice, macaques, and humans, exhibits hyaluronidase-like activity that facilitates this action [[8–11](#)]. Furthermore, a later study has demonstrated that a plasma membrane-associated hyaluronidase is localized at the posterior acrosomal region of equine sperm [[12](#)].

Having traversed the cumulus, the spermatozoon undergoes acrosome reaction and binds to the zona pellucida. Sperm–zona pellucida binding is mediated by specific sperm surface receptors. The primary ligand on the zona pellucida, ZP3, specifically binds to the plasma membrane of the acrosomal cap of the intact

sperm. The secondary zona ligand, ZP2, binds to the inner acrosomal membrane of the spermatozoon [[13–15](#)]. One of the inner acrosomal membrane sperm receptors was identified as acrosin [[16–18](#)]. In order to penetrate the zona pellucida, the spermatozoon utilizes enzymatic as well as mechanical mechanisms. Specific enzymes that are released by the acrosome-reacted spermatozoon allow the invasion of the zona pellucida by local degradation of its components [[19, 20](#)]. This enzymatic action is assisted by mechanical force generated by vigorous tail beatings that facilitate the penetration of the sharp sperm head [[18–22](#)].

Having penetrated the zona pellucida, the sperm crosses the perivitelline space and its head attaches to the egg's plasma membrane (oolemma). The key molecules that are crucial for the direct sperm–oolemma interaction in mammalian fertilization have been identified. The essential player on the sperms' side is known as the protein IZUMO1 and its prime binding partner on the oolemma is a folate receptor 4 named Juno [[23](#)].

Sperm head attachment to the oolemma is followed by its incorporation into the egg cytoplasm (ooplasm). Sperm incorporation is initiated by phagocytosis of the anterior region of its head followed by fusion of the head's posterior region along with the tail with the egg membrane [[24–26](#)]. The scientific efforts that have been invested by reproductive biologists in studying the process of gametogenesis and fertilization in animal models laid the groundwork for the design of *in vitro* procedures for assisted reproduction. These procedures that are successfully practiced at present in human patients essentially attempted to mimic the biological processes *in vivo*.

In vitro fertilization (IVF) regimens of treatment, which are continuously being improved, have allowed the birth of more than eight million babies all over the world, since the first IVF birth in 1978 of Louise Brown. One such improvement, which represents a major breakthrough in this area, is intracytoplasmic sperm injection (ICSI). Until 1992, most infertility failures originating from a severe male factor were untreatable. Micromanipulation techniques such as partial zona dissection [[27–30](#)] and sub-zonal sperm injection [[29, 31–35, 48](#)], designed to overcome the poor performance of sperm cells, did not result in a substantial improvement of the rate of success of *in vivo* fertilization. However, ICSI, which was established by the team led by Professor Van Steirteghen at the Free University in Brussels, Belgium, and initially reported by Palermo et al. [[35](#)], has generated dramatic progress [[36–39](#)]. The ICSI procedure involves the injection of a single sperm cell intracytoplasmically into an egg. Fertility failures associated with an extremely low sperm count were found to be successfully treated by this technique. Furthermore, as the sperm is microinjected into the ooplasm, it bypasses the passage through the zona pellucida and is not required to interact with the oolemma. Therefore, infertility problems that originate from faulty sperm–egg interaction may also be resolved by this IVF protocol of treatment.

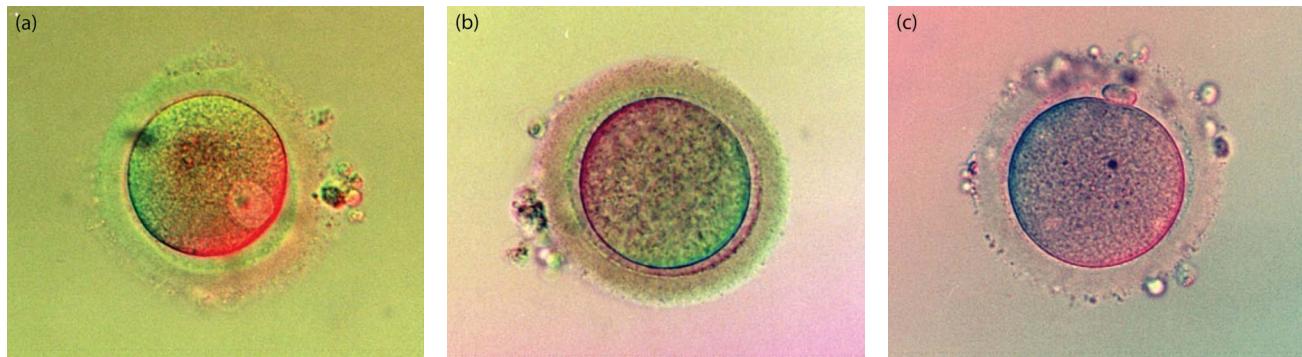


FIGURE 10.1 Morphological markers characterizing the meiotic status of oocytes. (a) Immature germinal vesicle (GV) oocyte: meiosis has not been re-initiated and the typical nuclear structure is visible. (b) Immature GV breakdown oocyte (metaphase I [MI]): meiosis has been re-initiated, the GV has disappeared, but the first polar body is still absent. (c) Mature oocyte (MII): the GV has disappeared and the first polar body has been extruded.

Handling of oocytes

Similar to conventional IVF, patients for ICSI undergo programmed induction of superovulation followed by scheduled oocyte retrieval ([Chapter 9](#)). Under all protocols of treatment, identification of the cumulus–oocyte complexes and evaluation of their maturity are carried out immediately after follicle aspiration, as described in [Chapter 9](#). However, unlike conventional IVF, in which intact mature cumulus–oocyte complexes are inseminated, cumulus cells that surround the eggs are removed before microinjection.

Denudation of the mature oocytes is an essential prerequisite for ICSI. Cumulus cells may block the injecting needle, thus interfering with oocyte microinjection. Furthermore, in the presence of the cumulus, visualization of the egg is very limited. Since only mature oocytes that have reached MII are suitable for ICSI, optimal optical conditions that allow the accurate assessment of the meiotic status of the oocytes are required. Oocyte maturation is determined morphologically by the absence of the GV and the presence of the first polar body. Good optical conditions are also necessary for the positioning of the mature oocyte in the right orientation for injection ([Chapter 13](#)). Preparation of the retrieved mature oocytes for ICSI should be carried out under conditions of constant pH of 7.3 and a stable temperature of 37°C. In order to maintain the appropriate pH, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES)-buffered, or 3-(*N*-morpholino) propanesulfonic acid (MOPS)-buffered culture media are used. The correct temperature is maintained during egg handling by the use of a microscope equipped with a heated stage. Most of the procedures are performed under paraffin/mineral oil that prevents evaporation of the medium and minimizes the fluctuations of both the pH and the temperature.

Temperature fluctuations that are likely to accompany the handling of eggs have been shown to be specifically detrimental for the microtubular system. Changes in spindle organization were observed in human mature oocytes cooled to room temperature for only 10 minutes. These changes included a reduction in spindle size, disorganization of microtubules within the spindle, and, in some cases, even a complete absence of microtubules [[40, 41](#)]. The susceptibility of the microtubules to temperature variations has been also shown in mature mouse oocytes [[42](#)]. Interference

with spindle organization can disturb the faithful segregation of the chromosomes, resulting in aneuploidy.

Laboratory procedures

Removal of the surrounding cumulus cells is accomplished by combined enzymatic and mechanical treatment carried out under a stereoscopic dissecting microscope. Different studies have tried to determine the pre-incubation time that is needed between egg retrieval and egg denudation and ICSI performance, however, no conclusive results were obtained.

A pre-incubation period of at least three hours between oocyte retrieval and removal of the cumulus cells to allow immature oocyte to resume meiosis *in vitro* was recommended by one study [[43](#)]. This recommendation was challenged by other studies which did not demonstrate differences in ICSI outcomes that correlated with the time interval between egg aspiration and microinjection [[44–46](#)]. On the other hand, pre-incubation time that exceeded nine hours resulted in embryos of lower quality [[44](#)]. This maximal time window was also challenged by a later study that showed no effect of OPU–ICSI time between 1 and 17 hours on fertilization rate, embryo quality, as well as pregnancy and live birth rates [[46](#)]. Garor et al. [[47](#)] demonstrated that it is not the time of oocyte denudation and injection that determines the ICSI outcome but, in fact, the time interval between human chorionic gonadotropin (hCG) administration and oocyte pickup (OPU). Specifically, fertilization along with pregnancy rates were significantly higher in IVF cycles in which the hCG–OPU interval exceeded 36 hours, regardless of the time of oocyte handling [[47](#)]. Since oocyte denudation cannot be carried out before some preliminary laboratory preparations that are described next are completed, a pre-incubation period of about 30 minutes is unavoidable. During this period, the retrieved mature cumulus–oocyte complexes are kept in the incubator at 37°C with 5%–6% CO₂ according to the recommendations of the culture media manufacturer.

Preliminary preparations for oocyte denudation Injecting dish

A special shallow Falcon dish (type 1006) is used for placing the denuded eggs. Nine small droplets of MOPS (3-(*N*-morpholino) propanesulfonic acid)-HEPES-buffered human tubal fluid culture

media (MHM; Irvine Scientific, CA, USA), containing 10% synthetic serum, 5–10 µL each, are arranged in a square of 3 × 3 within this dish. Two droplets of 5 µL of 10% polyvinylpyrrolidone (PVP), in which sperm will be placed, are added above the MHM droplets. This viscous solution slows sperm motility, facilitating sperm collection and control of the injecting pipette. It is recommended that one of the PVP droplets be elongated and flattened to ease sperm capture and visualization under the microscope. All droplets are then covered with either paraffin or mineral oil, and the dish is placed on the heated area in the hood to warm up before removal of the cumulus cells.

In cases of low sperm count and/or motility, sperm is placed in MHM droplets to allow better motility. In cases of very low sperm motility, the use of SpermMobile (GM501; Hamilton Thorne) is recommended. This “caffeine-like” solution increases cAMP levels in the sperm cells, stimulating their motility. This solution is added into one or more of the MHM droplets, to a final dilution of 1:20, according to the manufacturer’s protocol.

Enzymatic solution

Since hyaluronic acid is a major component of the mucified cumulus mass that surrounds the mature oocyte, hyaluronidase is employed for enzymatic removal of these cells (80 IU/mL; Sage In-Vitro Fertilization, Inc., Trumbull, CT). The high concentration of 760 IU/mL of hyaluronidase that was used initially [49] was found to induce parthenogenetic activation of the mature oocytes. A lower concentration of the enzyme, such as 80 IU/mL, which is commonly used, significantly decreased the rate of parthenogenesis [50]. According to our experience, hyaluronidase at a concentration of 60 IU/mL effectively denudes the oocytes. Further reduction of the enzyme concentration to as low as 10 IU/mL was also found to be sufficient [51].

Denuding dish

Two drops of ~100 µL of hyaluronidase solution and five drops of ~100 µL of MHM containing 10% serum covered with oil are placed in a 60-mm culture dish and covered with oil. The dish is then placed on the heated area in the hood to warm up.

Removal of the cumulus cells

Cumulus–oocyte complexes are transferred into the drop of hyaluronidase solution and repeatedly aspirated through a Pasteur pipette for up to 30–40 seconds. At this time, dissociation of the cells is initially observed. Further mechanical denudation is carried out in the enzyme-free MHM drops by repeated aspiration through commercially prepared stripper tips with decreasing inner diameters of 275, 170, and 140 µM and, when necessary, 135 µM. The oocytes are transferred through the drops of medium, until all coronal cells have been finally removed and all traces of enzyme have been washed off. This procedure is carried out very gently in order to avoid mechanical damage to the oocytes. Pricking of the oocyte has been shown to induce parthenogenetic activation [51, 52]. Finally, the denuded oocytes are placed in the droplets of the injecting dish and their morphology and meiotic status are evaluated. These procedures are performed on the heated area in the hood.

In order to maintain a temperature of 37°C during preparations and egg handling, the heated working area in the hood and microscope stage must be calibrated to a higher temperature (around 38°C).

In cases of extremely low sperm count or testicular sperm injection, oocytes must be kept in the incubator in CO₂-equilibrated

culture medium until a sufficient number of sperm cells have been collected.

Evaluation of denuded oocytes for ICSI

Oocytes are assessed for their maturation and for their morphology under an inverted microscope equipped with Nomarski differential interference contrast optics at 200× magnification. Only mature oocytes that resume their first meiotic division, reaching MII, are appropriate for ICSI. Evaluation of the meiotic status of the oocyte is based on morphological markers. In mature oocytes, the GV has disappeared and the first polar body is present and localized in the perivitelline space (Figure 10.1c).

Different studies have reported that in patients who undergo controlled ovarian stimulation (COS) 10%–20% of the retrieved oocytes have not resumed their meiotic division [53–57]. These oocytes can be divided into two categories: GV oocytes in which meiosis has not been re-initiated and the typical nuclear structure is visible (Figure 10.1a) and GVB oocytes in which meiosis has been re-initiated but did not proceed beyond the first metaphase (MI). In these oocytes, the GV has disappeared but the first polar body has not been extruded (Figure 10.1b). Oocytes in both of these categories are separated from the MII oocytes. MI oocytes are further incubated and those that extrude the first polar body within two to four hours are inseminated by ICSI [57, 58]. It has been reported that 74% of the MI oocytes completed meiosis *in vitro* within 20 hours after retrieval. This report did not find differences in the rates of fertilization and embryo development between these oocytes and other oocytes retrieved at MII. However, only sporadic pregnancies were achieved following the transfer of embryos obtained from fertilized MI oocytes that had matured *in vitro* [58, 59]. Another study demonstrated that 26.7% of MI oocytes extruded the first polar body *in vitro* within four hours. These oocytes were injected on the same day of follicle aspiration in parallel to the oocytes retrieved at MII. In this study, however, the MI oocytes that completed their maturation *in vitro* exhibited a lower fertilization rate, with no differences in embryo quality between oocytes that underwent maturation *in vitro* and those retrieved at MII. Similar to the previous study, only sporadic pregnancies were obtained following transfer of embryos developed from MI oocytes that had matured *in vitro* [60, 61]. Other studies support these observations, showing that although *in vitro*-matured (IVM) MI oocytes can be normally fertilized, the embryos derived from these oocytes rarely provide pregnancies [62, 63]. This is compatible with the findings that these embryos exhibit low morphological quality and a high rate of chromosomal abnormalities [56], along with the recent demonstration that blastulation rate is directly associated with oocyte maturation [57].

In patients with few MII oocytes, rescue of MI oocytes may increase the number of embryos for transfer; however, the chance of improving pregnancy rates by this procedure is minimal. Oocytes with GV require an overnight (30-hour) incubation in order to reach the MII stage. Only very sporadic pregnancies were reported from oocytes that were at the GV stage when retrieved during standard IVF treatment with COS [61, 62, 64]. Because of the very poor results, these GV oocytes are usually discarded. It has been demonstrated that GV oocytes that are retrieved after COS exhibit damaged DNA [65]. Therefore, only in cases in which very few or no MII oocytes are retrieved are the GV oocytes rescued for fertilization, provided they complete their maturation.

Incubation of the GV oocytes for 24 hours after OPU, to allow their spontaneous maturation, indeed resulted in a fertilization

rate of 64%, however, the embryos developed from these oocytes were of a very poor quality and usually aneuploid [66].

Different COS protocols may result in follicular asynchrony and variations in oocyte number and quality [67]. Furthermore, during aspiration, oocytes are collected from heterogeneous groups of follicles, leading to aspiration of mature MII oocytes or meiotically immature MI or GV oocytes [68]. As shown in **Figure 10.3a**, using a modified polarized light microscope, the “PolScope,” it is possible to detect oocytes with PB in which the second meiotic spindle has not yet been formed and the first meiotic spindle (mid-body) is still observed. This indicates that the presence of a PB does not necessarily represent a fully mature fertilizable egg. A high proportion of immature oocytes during OPU, may reflect lower quality of the retrieved MII oocytes. Such MII oocytes result in a lower success rate as compared to MII oocytes in cycles with no GV/MI oocytes [57, 65].

Immature GV oocytes can also be retrieved from the small (3–13 mm) ovarian follicles present in unstimulated patients [69–72]. Although these oocytes were not exposed to LH *in vivo*, they are apparently meiotically competent and can be expected to mature spontaneously *in vitro* and produce normal eggs. In 1998, Goud et al. showed a fertilization rate of 46% by ICSI of such IVM GV oocytes [72] resulting in a few pregnancies. However, as more experience is gained in handling immature oocytes, success rates are increasing worldwide [73, 74]. Later studies demonstrated that hCG administration before oocyte retrieval from the small follicles accelerates their *in vitro* maturation, resulting in better embryonic development and leading to higher pregnancy rates. It was further demonstrated that administration of low doses of follicle-stimulating hormone (FSH) before hCG priming enables the

retrieval of *in vivo*-matured oocytes (MII) from the small follicles (<10 mm). Such oocytes have a higher potential to develop into good-quality embryos than IVM oocytes, achieving even higher pregnancy rates [75].

Nowadays, IVM is characterized by minimal administration of FSH or human menopausal gonadotropin (hMG) prior to oocyte retrieval, without triggering of ovulation by hCG. Using new IVM culture systems the efficacy of IVM has significantly improved, reaching a live birth rate of 40% in different clinics worldwide (reviewed by Young et al. [76]). Advantages of IVM over *in vitro* fertilization (IVF) include mild or no hyperstimulation, lower medication costs, and less patient burden. It is clinically beneficial for patients who suffer previously from ovarian hyperstimulation syndrome and patients who must undergo immediate chemotherapy and need fertility preservation.

In addition to the meiotic status, the morphology of the oocytes is also evaluated before ICSI. The various morphological defects may be manifested by an amorphous shape of the oocyte, enlargement of or granularity in the perivitelline space, inclusions, vacuolization, granularity, and dark colour of the cytoplasm, changes in the colour and construction of the zona pellucida, and changes in the shape and size of the polar body (**Figure 10.2**). Most defective oocytes exhibit more than one of the aforementioned abnormalities. All these observations should be recorded and may help in later analysis of the fertilization rate, embryo development, and pregnancy outcomes after ICSI. The correlations between egg morphology and the rates of fertilization, embryo quality, and pregnancy after ICSI have been extensively studied. Most of the studies reported that abnormal egg morphologies of patients undergoing ICSI are associated with a lower rate of fertilization,

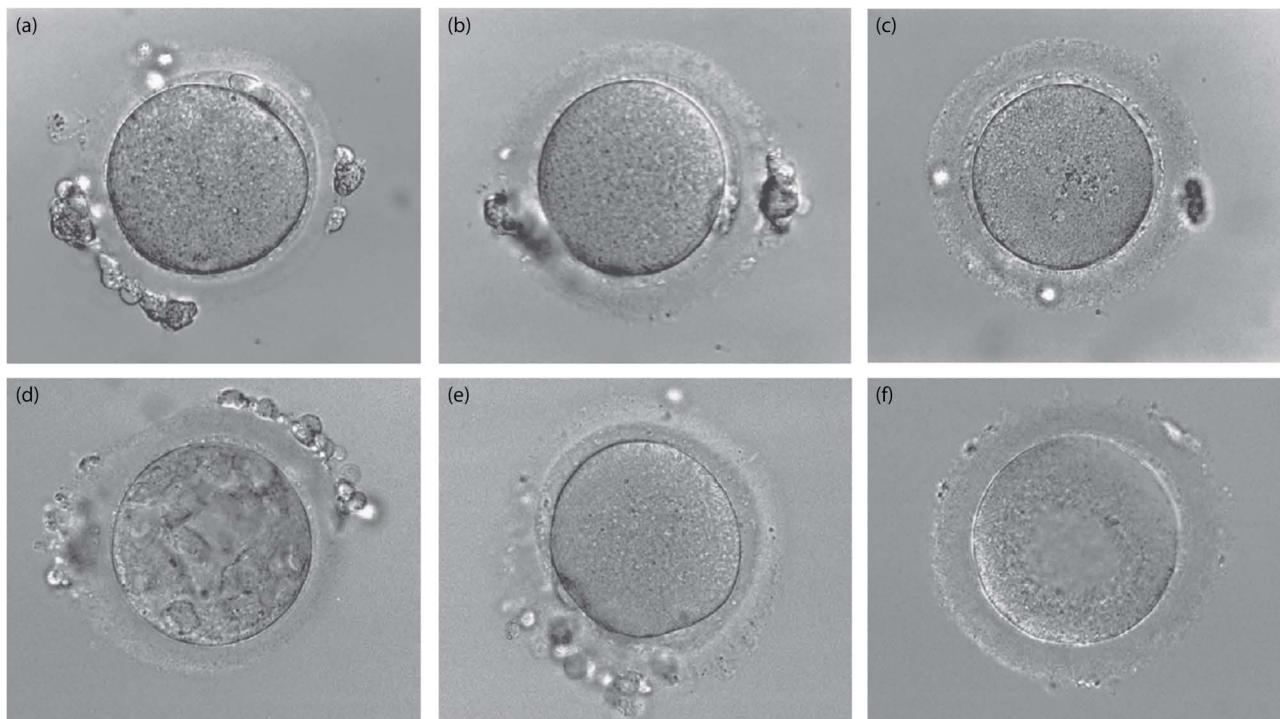


FIGURE 10.2 Various morphological abnormalities exhibited by oocytes. (a) Granulated perivitelline space; (b) a fragmented polar body; (c) thickened and dark-coloured zona pellucida; (d) cytoplasmic inclusions; (e) enlarged and granulated perivitelline space; and (f) a large cytoplasmic vacuole.

embryos of poor quality, and, consequently, lower pregnancy rates [77–79]. Other studies demonstrated successful fertilization and normal early embryo development in microinjected eggs with defective morphologies, such as large perivitelline space, cytoplasmic vacuoles, or a fragmented polar body [80–83]. However, the transfer of these seemingly normal embryos resulted in a poor implantation rate [79] and a high incidence of early pregnancy loss [81]. The use of a triple marker model of polar body shape, size of the perivitelline space, and cytoplasmic inclusions has been suggested by Xia for human oocyte grading [78]. This laboratory reported that the evaluation of oocyte quality based on these criteria correlated well with the rate of fertilization and with embryo quality after ICSI.

As mentioned previously in this chapter, the integrity of the meiotic spindle in MII oocytes is crucial for fertilization capacity and embryo development. Therefore, in addition to the aforementioned features of the oocyte, the morphology of the spindle may serve as a reliable marker for predicting its potential for normal fertilization and embryonic development [84]. The “PolScope” mentioned earlier, which is a modification of the polarized light microscope, equipped with novel image-processing software [85], has emerged as a non-invasive tool to view the meiotic spindle in living oocytes. The image of the spindle is based on the highly birefringent characteristic of the microtubule filaments under a polarization microscope. Meiotic spindle in the cytoplasm of living oocytes cannot be visualized with the standard light microscope that is routinely used in IVF laboratories. Due to this obvious advantage, the use of PolScope has been implemented in several IVF units worldwide [41, 84, 86–88]. Examination of human oocytes by PolScope has indeed demonstrated that the absence or abnormal morphology of the spindle is highly correlated with lower fertilization rates and impaired embryonic development [86, 87, 89]. Furthermore, spindle assessment with the PolScope has been shown to facilitate the selection of embryos with high implantation potential for transfer [84]. It was demonstrated that a spindle can be detected in 75% of the oocytes, and its detection directly correlates with higher chances for achieving pregnancy. Furthermore, the number of spindle-positive oocytes decreases with increasing age of patients [88]. A recent study demonstrated that the size of the spindle can be measured by the PolScope, demonstrating that 90–120 μm^2 is the optimal

size that predicts higher fertilization, blastocyst formation, and clinical pregnancy rates [90].

The use of the PolScope has also indicated that polar body position does not always accurately predict spindle location. In MII oocytes, the second meiotic spindle is expected to be adjacent to the first polar body (Figure 10.3b), making the first polar body a marker for appropriate orientation of the ICSI micropipette to avoid interference with chromosome alignment. However, observations by Silva et al. [86] and ourselves that the meiotic spindle is not always adjacent to the polar body (Figure 10.3c) have made use of the PolScope even more valuable. In the study of Konc et al. [88], of 320 tested oocytes, 66% exhibited a spindle that was positioned at a 45° angle from the PB. Furthermore, as mentioned earlier, in those oocytes that have not yet completed the formation of the first polar body, the PolScope can detect the presence of microtubules in the mid-body, suggesting that the second meiotic spindle has not yet been fully organized (Figure 10.3a), and ICSI should be postponed.

Appropriate ovarian stimulation protocols normally provide functional, fertilizable mature oocytes, while oocytes of poor quality may represent a disturbed hormonal balance. For example, exposure to high dosage of hMG has been shown to be associated with granularity of the perivitelline space [55]. Moreover, an extended exposure to high doses of this hormone may lead to the senescence of the mature oocyte before retrieval. As previously mentioned, oocyte maturation and ovulation are both stimulated by LH. However, studies have shown that the ovulatory response is less sensitive to this gonadotropin, requiring higher concentrations of the hormone [91]. Therefore, the relatively high concentration of LH in hMG effectively promotes oocyte maturation, but is insufficient to stimulate ovulation. Delayed administration of hCG in these patients entraps the mature oocytes in the follicle, leading to oocyte aging. One notable morphological marker in this case is the fragmentation of the first polar body [92]. The presence of aged oocytes can also explain the decreased quality of oocytes and lower fertilization rate in polycystic ovarian syndrome patients [93] who exhibit relatively high serum concentrations of LH throughout their menstrual cycle [94]. Nowadays, pure FSH preparations (recombinant FSH) are widely used for the stimulation of follicular growth and development. However, it has been demonstrated that introducing low concentrations of

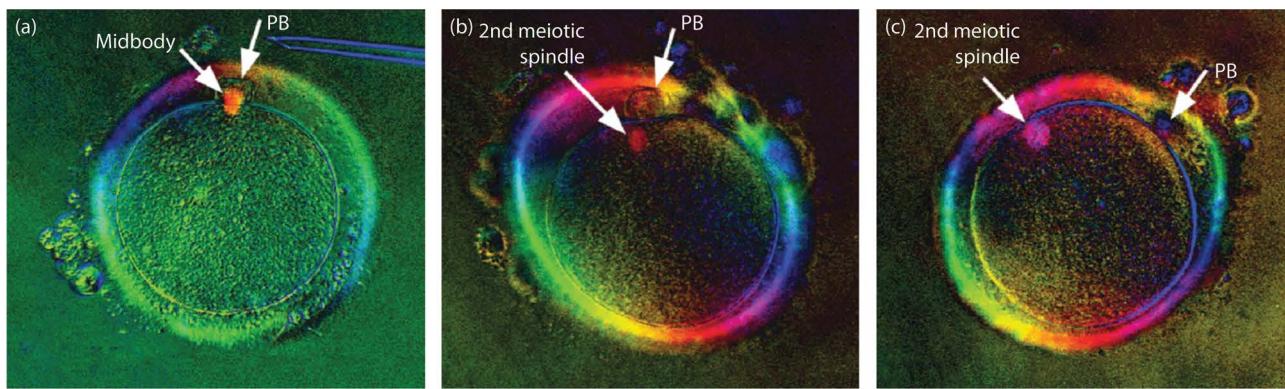


FIGURE 10.3 Microtubule images in metaphase II (MII) human oocytes. (a) Microtubules of the mid-body extending from the cytoplasm into the first polar body (PB). (b) Microtubules of the second meiotic spindle located adjacent to the PB. (c) Microtubules of the second meiotic spindle at a distal location from the PB.

LH (recombinant LH) in addition to FSH significantly improves IVF outcomes [95].

The accumulated experience worldwide indicates that the response to hormonal stimulation varies substantially among women and these individual variations are dependent not only on the stimulation protocol but also on the genetic background. It has been shown that the expression of different genes in granulosa cells, such as ADAMTS1 and HSPG2, is directly associated with oocyte quality and IVF outcome [96, 97]. These genes may serve as genetic markers for predicting ovulation response, facilitating the optimization of the stimulation regimen for each patient individually.

Epilogue

A baby girl is born with her ovaries containing about two million oocytes, all of which arrested at the prophase of the first meiotic division. This pool of oocytes remains dormant throughout childhood until the onset of puberty. In sexually mature females, at each cycle, one such “sleeping beauty” is kissed by the LH “prince” and awakened to continue its meiotic division. Once maturation has been completed, the oocyte is released from the ovarian follicle into the fallopian tube, a site at which it will eventually meet the spermatozoon and undergo fertilization. Hormonal stimulation protocols are designed to mimic the natural events that lead to the production of mature oocytes. In IVF patients, these oocytes are aspirated from the ovarian follicles prior to ovulation and allowed to meet the sperm cells in the Petri dish. A higher scale of assistance, designed to overcome the poor performance of spermatozoa, is offered by ICSI. The information regarding oocyte handling for this later modification of the classical IVF protocol has been summarized in this chapter.

APPENDIX

Laboratory protocol

The following protocol is used in our laboratory.

- Preliminary preparations for oocyte denudation
 1. Injecting dish: the droplets may be placed on the dish in any arrangement the laboratory prefers. Our laboratory recommends the following layout. Place nine droplets, 5 µL each, of MHM containing 10% serum, arranged in a 3 × 3 square within a shallow Falcon dish (type 1006). Place two additional droplets of 5 µL of 7%–10% PVP solution where the sperm will be placed. One of the PVP droplets will be elongated and flattened. Cover with oil. Place the dish on the heated area in the hood to warm up. In cases of extremely low sperm counts and/or motility, MHM as well as more than one PVP droplet can be used for sperm. In cases of very low sperm motility, add SpermMobile solution (GM501, Hamilton Thorne) into one or more of the MHM droplets, to a final dilution of 1:20, according to the manufacturer's protocol.
 2. Denuding dish: place a drop of 100 µL of hyaluronidase solution 80 IU/mL (Sage) and five 100 µL drops of MHM containing 10% serum in an embryo-tested 60-mm culture dish. Cover with oil and place on the heated area in the hood to warm up.
 3. Stripper tips: Prepare stripper tips with inner diameters of 275, 170, and 140 µM.

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- Removal of the cumulus cells
 1. Place the cumulus–oocyte complexes into the drop of the hyaluronidase solution (up to five complexes at a time) and aspirate repeatedly through a Pasteur pipette for up to 40 seconds.
 2. Transfer the cumulus–oocyte complexes to a drop of MHM containing 10% serum and aspirate repeatedly through a 275 µM diameter stripper tip. Continue aspirating with 170 and 140 µM tip while passing the oocytes through the other four drops of the medium, until all corona cells have been totally removed.
 3. Transfer the denuded oocytes to the MHM droplets in the injecting dish. It is recommended to place no more than one oocyte in each droplet.
- Microscopic evaluation
 1. Place the injecting dish containing the oocytes on the heated stage of an inverted microscope equipped with differential interference contrast.
 2. Evaluate oocyte morphology and meiotic status at 200× magnification.

References

1. Dekel N, Aberdam E, Goren S, Feldman B, Shalgi R. Mechanism of action of GnRH-induced oocyte maturation. *J Reprod Fert*. 1989;37:319–27.
2. Lindner HR, Tsafriri A, Lieberman ME, et al. Gonadotropin action on cultured Graafian follicles: Induction of maturation division of the mammalian oocyte and differentiation of the luteal cell. *Recent Prog Horm Res*. 1974;30:79–138.
3. Dekel N. Hormonal control of ovulation. In: *Biochemical Action of Hormones*. Litwack G (ed.). Orlando, FL: Academic Press, pp. 57–90, 1986.
4. Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ. FSH induced expansion of the mouse cumulus oophorus *in vitro* is dependent upon a specific factor(s) secreted by the oocyte. *Dev Biol*. 1990;138:16–25.
5. Salustri A, Yanagishita M, Hascall VC. Mouse oocytes regulate hyaluronic acid synthesis and mucification by FSH-stimulated cumulus cells. *Dev Biol*. 1990;138:26–32.
6. Vanderhyden BC, Caron PJ, Buccione R, Eppig JJ. Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. *Dev Biol*. 1990;140:307–17.
7. Vanderhyden BC. Species differences in the regulation of cumulus expansion by an oocyte secreted factor(s). *J Reprod Fertil*. 1993;98:219–27.
8. Lin Y, Mahan K, Lathorp W, Myles D, Primakoff P. A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *J Cell Biol*. 1994;125:1157–63.
9. Cherr G, Meyers S, Yudin A, et al. The PH-20 protein in cynomolgus macaque spermatozoa: Identification of two different forms exhibiting hyaluronidase activity. *Dev Biol*. 1996;175:142–53.
10. Oversreet J, Lin Y, Yudin A, et al. Location of the PH-20 protein on acrosome-intact and acrosome reacted spermatozoa of cynomolgus macaques. *Biol Reprod*. 1995;52:105–14.
11. Sabeur K, Cherr G, Yudin A, et al. The PH-20 protein in human spermatozoa. *J Androl*. 1997;18:151–8.
12. Meyers SA, Rosenberger AE. A plasma membrane associated hyaluronidase is localized to the posterior acrosomal region of stallion sperm and is associated with spermatozoal function. *Biol Reprod*. 1999;61:444–51.

13. Bleil JD, Wasserman PM. Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *J Cell Biol.* 1986;102:1363–71.
14. Beaver EL, Friend DS. Morphology of mammalian sperm membranes during differentiation, maturation, and capacitation. *J Electr Microscop Tech.* 1990;16:281–97.
15. Mortillo S, Wasserman PM. Differential binding of gold-labeled zona pellucid glycoproteins mZP2 and mZP3 to mouse sperm membrane compartments. *Development.* 1991;113:141–9.
16. Jones R. Interaction of zona pellucida glycoproteins, sulphated carbohydrates and synthetic polymers with proacrosin, the putative egg-binding protein from mammalian spermatozoa. *Development.* 1991;111:1155–63.
17. Urch UA, Patel H. The interaction of boar sperm proacrosin with its natural substrate, the zona pellucida, and with polysulphated polysaccharides. *Development.* 1991;111:1165–72.
18. Yanagimachi R. *Fertilization and Embryonic Development In Vitro.* New York, NY: Plenum Press, 1981.
19. Brown CR, Cheng WTK. Limited proteolysis of the porcine zona pellucida by homologous sperm acrosin. *J Reprod Fertil.* 1985;74:257–60.
20. Dunbar BS, Prasad SV, Timmons TM. *Comparative Overview of Mammalian Fertilization.* New York, NY: Plenum Press, 1991.
21. Dunbar BS, Budkiewicz AB, Bundman DS. Proteolysis of specific porcine zona pellucida glycoproteins by boar acrosin. *Biol Reprod.* 1985;32:619–30.
22. Yanagimachi R. Time and process of sperm penetration into hamster ova *in vivo* and *in vitro*. *J Reprod Fertil.* 1966;11:359–70.
23. Bianchi E, Doe B, Goulding D, Wright G. Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature.* 2014;508:483–7.
24. Phillips DM, Shalgi RM. Sperm penetration into rat ova fertilized *in vivo*. *J Exp Zool.* 1982;221:373–8.
25. Shalgi R, Phillips D. Mechanics of sperm entry in cycling hamsters. *J Ultrastruct Res.* 1980;71:154–61.
26. Shalgi R, Phillips DM, Jones R. Status of the rat acrosome during sperm–zona pellucida interactions. *Gamete Res.* 1989;22:1–13.
27. Cohen J, Malter H, Fehilly C, et al. Implantation of embryos after partial opening of oocyte zonal pellucid to facilitate sperm penetration. *Lancet.* 1988;2:162.
28. Cohen J, Malter H, Wright G, et al. Partial zona dissection of human oocytes when failure of zona pellucida is anticipated. *Hum Reprod.* 1989;4:435–42.
29. Cohen J, Talanski BE, Malter HM, et al. Microsurgical fertilization and teratozoospermia. *Hum Reprod.* 1991;6:118–23.
30. Tucker MJ, Bishop FM, Cohen J, et al. Routine application of partial zona dissection for male factor infertility. *Hum Reprod.* 1991;6:676–81.
31. Laws-King A, Trounson A, Sathananthan H, et al. Fertilization of human oocytes by microinjection of single spermatozoon under zona pellucida. *Fertil Steril.* 1987;48:637–42.
32. Ng SC, Bongso A, Ratnam SS, et al. Pregnancy after transfer sperm under zona. *Lancet.* 1988;2:790.
33. Bongso TA, Sathananthan AH, Wong C, et al. Human fertilization by microinjection of immotile spermatozoa. *Hum Reprod.* 1989;4:175–9.
34. Palermo G, Joris H, Devoroey P, et al. Induction of acrosome reaction in human spermatozoa used subzonal insemination. *Hum Reprod.* 1992;7:248–54.
35. Palermo G, Joris H, Devoroey P, et al. Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. *Lancet.* 1992;340:17–8.
36. Palermo G, Joris H, Devoroey P, et al. Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Fertil Steril.* 1993;59:826–35.
37. Van Steirteghem AC, Liu J, Nagy Z, et al. Use of assisted fertilization. *Hum Reprod.* 1993;8:1784–5.
38. Van Steirteghem AC, Liu J, Joris H, et al. Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of second series of 300 consecutive treatment cycles. *Hum Reprod.* 1993;8:1055–60.
39. Van Steirteghem AC, Nagy Z, Joris H, et al. High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum Reprod.* 1993;8:1061–6.
40. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril.* 1990;54:102–8.
41. Wang WH, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindle in living human oocytes after cooling–rewarming observed using polarized microscopy. *Hum Reprod.* 2001;16:2374–8.
42. Magistrini M, Szollosi D. Effects of cold and isopropyl-n-phenylcarbamate on the second meiotic spindle of mouse oocytes. *Eur J Cell Biol.* 1980;22:699–707.
43. Rienzi L, Ubaldi F, Anniballo R, Cerulo G, Greco E. Preincubation of human oocytes may improve fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod.* 1998;13:1014–9.
44. Yanagida K, Yazawa H, Katayose H, et al. Influence of preincubation time on fertilization after intracytoplasmic sperm injection. *Hum Reprod.* 1998;13:2223–6.
45. Van de Velde H, De Vos A, Joris H, Nagy ZP, Van Steirteghem AC. Effect of timing of oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod.* 1998;13:3160–4.
46. Bárcena P, Rodríguez M, Obradors A, Vernaeve V, Vassena R. Should we worry about the clock? Relationship between time to ICSI and reproductive outcomes in cycles with fresh and vitrified oocytes. *Hum Reprod.* 2016;31(6):1182–91.
47. Garor R, Shufaro Y, Kotler N, Shefer D, Krasilnikov N, Ben-Haroush A, Pinkas H, Fisch B, Sapir O. Prolonging oocyte *in vitro* culture and handling time does not compensate for a shorter interval from human chorionic gonadotropin administration to oocyte pickup. *Fertil Steril.* 2015;103:72–5.
48. Palermo G, Joris H, Derde MP, et al. Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Fertil Steril.* 1993;59:826–35.
49. Joris H, Nagy Z, Van de Velde H, De Vos A, Van Steirteghem A. Intracytoplasmic sperm injection: Laboratory set-up and injection procedure. *Hum Reprod.* 1998;13(Suppl 1):76–86.
50. Van de Velde H, Nagy ZP, Joris H, De Vos A, Van Steirteghem AC. Effects of different hyaluronidase concentrations and mechanical procedures for cumulus cell removal on the outcome of intracytoplasmic sperm injection. *Hum Reprod.* 1997;12:2246–50.
51. Iritani A. Micromanipulation of gametes for *in vitro* assisted fertilization. *Mol Reprod Dev.* 1991;28:199–207.
52. Flaherty SP, Payne D, Swann NG, et al. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod.* 1995;10:2629–32.
53. Junca AM, Mandelbaum J, Belaisch-Allert J, et al. Oocyte maturity and quality: Value of intracytoplasmic sperm injection. *Fertility of microinjected oocytes after *in vitro* maturation. Contracept Fertil Sex.* 1995;23:463–645.
54. Mandelbaum J, Junca AM, Balaisch-Allert J, et al. Oocyte maturation and intracytoplasmic sperm injection. *Contracept Fertil Sex.* 1996;24:534–8.
55. Hassan-Ali H, Hisham-Saleh A, El-Gezeiry D, et al. Perivitelline space granularity: A sign of human menopausal gonadotropin overdose in intracytoplasmic sperm injection. *Hum Reprod.* 1998;13:4325–30.
56. De Vos A, Van de Velde H, Joris H, Van Steirteghem A. In-vitro matured metaphase-I oocytes have a lower fertilization rate but similar embryo quality as mature metaphase-II oocytes after intracytoplasmic sperm injection. *Hum Reprod.* 1999;14:1859–63.

57. Bilibio JP, Lorenzoni PL, Meireles AJC, Maciel Y, Sales P, Costa do Nascimento F. The usefulness of metaphase I oocytes in women who undergo controlled ovarian hyperstimulation for intracytoplasmic sperm injection. *JBRA Assist Reprod.* 2021;25(1):115–21.
58. Strassburger D, Goldstein A, Friedler S, et al. The cytogenetic constitution of embryos derived from immature (metaphase I) oocytes obtained after ovarian hyperstimulation. *Fertil Steril.* 2010;94:971–8.
59. Coetzee K, Windt ML. Fertilization and pregnancy using metaphase I oocytes in an intracytoplasmic sperm injection program. *J Assist Reprod Genet.* 1996;13:768–71.
60. Strassburger D, Friedler S, Raziel A, et al. The outcome of ICSI of immature MI oocytes and rescued *in vitro* matured MII oocytes. *Hum Reprod.* 2004;19:1587–90.
61. Nagy ZP, Cecile J, Liu J, et al. Pregnancy and birth after intracytoplasmic sperm injection of *in vitro* matured germinal-vesicle stage oocytes: Case report. *Fertil Steril.* 1996;65:1047–50.
62. Jaroudi KA, Hollanders JMG, Sieck UV, et al. Pregnancy after transfer of embryos which were generated from *in-vitro* matured oocytes. *Hum Reprod.* 1997;12:857–9.
63. Liu J, Katz E, Garcia JE, et al. Successful *in vitro* maturation of human oocytes not exposed to human chorionic gonadotropin during ovulation induction, resulting in pregnancy. *Fertil Steril.* 1997;67:566–8.
64. Menezo YJ, Nicollet B, Rollet J, Hazout A. Pregnancy and delivery after *in vitro* maturation of naked ICSI-GV oocytes with GH and transfer of a frozen thawed blastocyst: Case report. *J Assist Reprod Genet.* 2006;23:47–9.
65. Astbury P, Subramanian GN, Greaney J, Roling C, Irving J, Homer HA. The presence of immature GV– stage oocytes during IVF/ICSI is a marker of poor oocyte quality: A pilot study. *Med Sci.* 2020;8:4: 1–11.11.
66. Vellez LT, Brogliato C, Berton CZ, Yoshida IH, Barbosa CP, Cordts B. ICSI in late matured oocytes, is it worth it? Study with laboratory, clinical and genetic evaluation results. *JBRA Assist Reprod.* 2020;24(2):173–4.
67. Jungheim ES, Meyer M, Broughton DE. Best Practices for controlled ovarian stimulation in IVF. *Semin Reprod Med.* 2015;33:77–82.
68. Stouffer RL, Zelinski-Wooten MB. Overriding follicle selection in controlled ovarian stimulation protocols: Quality vs quantity. *Reprod Biol Endocrinol.* 2004;2:32.
69. Edrishinge WR, Junk SM, Matson PL, Yovich JL. Birth from cryopreserved embryos following *in-vitro* maturation of oocytes and intracytoplasmic sperm injection. *Hum Reprod.* 1997;12:1056–8.
70. Trounson A, Anderiesz C, Jones GM, et al. Oocyte maturation. *Hum Reprod.* 1998; 13(Suppl 3): 52–62; discussion 71–5.
71. Russel JB. Immature oocyte retrieval with *in-vitro* maturation. *Curr Opin Obstet Gynecol.* 1999;11:289–96.
72. Goud PT, Goud AP, Qian C, et al. *In-vitro* maturation of human germinal vesicle stage oocytes: Role of cumulus cells and epidermal growth factor in the culture medium. *Hum Reprod.* 1998;13:1638–44.
73. Mikkelsen AL. Strategies in human *in-vitro* maturation and their clinical outcome. *Reprod Biomed Online.* 2005;10:593–9.
74. Al-Sunaidi M, Tulandi T, Holzer H, et al. Repeated pregnancies and live births after *in vitro* maturation treatment. *Fertil Steril.* 2007; 87: 1212.e9–12.
75. Weon-Young S, Seang LT. Laboratory and embryological aspect of hCG-primed *in vitro* maturation cycles for patients with polycystic ovaries. *Hum Reprod.* 2010;6:675–89.
76. Vuong LN, Ho TM, Gilchrist RB, Smitz J. The place of in vitro maturation in assisted reproductive technology. *Fertil. Rerprod.* 2019;1(1):11–5.
77. Sousa M, Tesarik J. Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. *Hum Reprod.* 1994;9:2374–80.
78. Xia P. Intracytoplasmic sperm injection: Correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality. *Hum Reprod.* 1997;12:1750–5.
79. Loutradis D, Drakakis P, Kallianidis K, et al. Oocyte morphology correlates with embryo quality and pregnancy rate after intracytoplasmic sperm injection. *Fertil Steril.* 1999;72:240–4.
80. De Sutter P, Dozortsev D, Qian C, Dhont M. Oocyte morphology does not correlate with fertilization rate and embryo quality after intracytoplasmic sperm injection. *Hum Reprod.* 1996;11:595–7.
81. Alikani M, Palermo G, Adler A, et al. Intracytoplasmic sperm injection in dysmorphic human oocytes. *Zygote.* 1995;3:283–8.
82. Serhal PF, Ranieri DM, Kinis A, et al. Oocyte morphology predicts outcome of intracytoplasmic sperm injection. *Hum Reprod.* 1997;12:1267–70.
83. Balaban B, Urman B, Sertac A, et al. Oocyte morphology does not affect fertilization rate, embryo quality and implantation rate after intracytoplasmic sperm injection. *Hum Reprod.* 1998;13:3431–3.
84. Kilani S, Cooke S, Tilia L, Chapman M. Does meiotic spindle normality predict improved blastocyst development, implantation and live birth rates? *Fertil Steril.* 2011;96:389–93.
85. Oldenbourg R, Mei G. New polarized light microscope with precision universal compensator. *J Microsc.* 1995;180:140–7.
86. Silva CP, Kommineni K, Oldenbourg R, Keefe DL. The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. *Fertil Steril.* 1999;71:719–21.
87. Wang WH. Spindle observation and its relationship with fertilization after ICSI in living human oocytes. *Fertil Steril.* 2001;75:348–53.
88. Konc J, Kanyo K, Cseh S. Visualization and examination of the meiotic spindle in human oocytes with polscope. *J Assist Reprod Gen.* 2004;21(10):349–53.
89. Moon JH, Hyun CS, Lee SW, et al. Visualization of the metaphase II meiotic spindle in living human oocytes using the PolScope enables the prediction of embryonic developmental competence after ICSI. *Hum Reprod.* 2003;18:817–20.
90. Tomari H, Honjo K, Kunitake K, Aramaki N, Kuhara S, Hidaka N, Nishimura K, Nagata Y, Horiuchi T. Meiotic spindle size is a strong indicator of human oocyte quality. *Reprod Med Biol.* 2018;17:268–74.
91. Dekel N, Ayalon D, Lewysohn O, et al. Experimental extension of the time interval between oocyte maturation and ovulation: Effect on fertilization and first cleavage. *Fertil Steril.* 1995;64:1023–8.
92. Eichenlaub-Ritter U, Schmiady H, Kentenich H, et al. Recurrent failure in polar body formation and premature chromosome condensation in oocytes from a human patient: Indicators of asynchrony in nuclear and cytoplasmic maturation. *Hum Reprod.* 1995;10:2343–9.
93. Aboulghar MA, Mansour RT, Serour GI, Ramzy AM, Amin YM. Oocyte quality in patients with severe ovarian hyperstimulation syndrome. *Fertil Steril.* 1997;68:1017–21.
94. Shoham Z, Jacobs HS, Insler V. Luteinizing hormone: Its role, mechanism of action, and detrimental effects when hypersecreted during the follicular phase. *Fertil Steril.* 1993;59:1153–61.
95. Franco JC Jr, Baruffi RLR, Oliveira JBA, et al. Effects of recombinant LH supplementation to recombinant FSH during induced ovarian stimulation in the GnRH agonist protocol: A matched case-control study. *Reprod Biol Endocrinol.* 2010;94:971–8.
96. Xiao S, Li Y, Li T, Chen M, Xu Y, Wen Y, Zhou C. Evidence for decreased expression of ADAMTS-1 associated with impaired oocyte quality in PCOS patients. *J Clin Endocrinol Metab.* 2014;99(6):E1015–21.
97. Ma Y, Jin J, Tong X, Yang W, Ren P, Dai Y, Pan Y, Zhang Y, Zhang S. ADAMTS1 and HSPG2 mRNA levels in cumulus cells are related to human oocyte quality and controlled ovarian hyperstimulation outcomes. *J Assist Reprod Gen.* 2020;37:657–67.

11

USE OF IN VITRO MATURATION OF OOCYTES IN A CLINICAL SETTING

What Is Its Role in ART?

Tuong M. Ho and Lan N. Vuong

Introduction

In vitro maturation (IVM) of oocytes is an assisted reproductive technology (ART) that involves collection of immature cumulus–oocyte complexes (COCs) at the prophase I stage, followed by maturation to metaphase II (MII) stage *in vitro* [1–4]. In contrast to *in vitro* fertilization (IVF), the typical IVM treatment cycle is characterized by minimal administration of follicle-stimulating hormone (FSH) or human menopausal gonadotropin (hMG) prior to oocyte retrieval, and no triggering of ovulation using human chorionic gonadotropin (hCG) [5, 6]. This chapter highlights the technical aspects of IVM and its current practice in the ART field, and includes a discussion about barriers to the use of IVM and approaches to overcome these obstacles.

History and development of human IVM

Human IVF and different ARTs have been developed over the last 40 years and have helped several millions of infertile couples to overcome childlessness. In fact, initial attempts to use IVM for human oocytes started early on to provide human oocytes for human IVF experiments because it was impossible to collect *in vivo* matured human oocytes at that time [1, 7, 8]. Edwards et al. achieved their landmark work in human IVF with human IVM oocytes [9]. Therefore, human IVF was actually developed based on the very early success of harvesting and IVM of human oocytes [9].

The first human live birth resulting from IVF was produced by natural cycle IVF, in which mature human oocytes were collected from pre-ovulatory follicles [10]. Subsequently, different ovarian stimulation protocols have been applied to increase the number of oocytes retrieved to improve the number embryos achieved and the chance of successful pregnancy per treated IVF cycle [11–14]. However, use of different ovarian stimulation protocols makes IVF more complicated and more expensive.

Despite its initial role in IVF research, there was almost no further development of IVM until the first baby conceived after IVM of immature oocytes derived from oocyte donors was reported by Cha and colleagues in 1991 [4]. Since then, the number of publications relating to IVM has increased, with the investigation of different protocols and a growing body of data reported (Table 11.1). Thus, IVM started to attract more interest from the ART community.

In 1994, Trounson et al. reported a case series describing babies born from immature oocytes collected from women with PCOS [15]. The trend to using a protocol for IVM in which different types of gonadotropins were administered before immature oocytes were recovered from small follicles started by the end of 1990s [16–18]. During the last two decades, IVM has been

utilized for patients with PCOS to reduce the health risks associated with ovarian stimulation, for fertility preservation, or just as an alternative, more user-friendly approach to ART [19]. Recently, human IVM has been considered as a potential treatment for fertility preservation in women with cancer for whom ovarian stimulation is contraindicated or when the patient does not have enough time for ovarian stimulation due to the urgency of cancer treatment [20, 21]. The European Society of Human Reproduction and Embryology (ESHRE) Guideline on Female Fertility Preservation published in 2020 suggests IVM as a fertility preservation technique [22]. Over the last five years, a new biphasic IVM culture system has been developed to improve the efficacy of IVM. Recent data from this new IVM culture system, called capacitation (CAPA) IVM, shows promising results [23, 24].

In 2021, the Practice Committees of the American Society for Reproductive Medicine (ASRM), the Society of Reproductive Biologists and Technologists, and the Society for Assisted Reproductive Technology (SART) published a landmark document that presented an overview of published evidence supporting the conclusion that IVM should no longer be considered an experimental technique [3]. The potential for wider clinical application of IVM was suggested [3].

Advantages and disadvantages of IVM

IVM has several advantages over IVF. Firstly, IVM uses mild or no stimulation and therefore the risk of ovarian hyperstimulation syndrome (OHSS) in patients with polycystic ovaries (PCO) or PCOS is largely eliminated [2, 25, 26]. Another advantage is lower medication costs because the requirement for expensive gonadotropins is reduced [2, 27, 28]. Finally, IVM is more convenient for patients because of a much lower monitoring burden (frequent ultrasounds and blood tests are not required), reduced stress, and fewer patient observations [2, 27, 28]. Despite these advantages, IVM was not widely adopted by the profession because clinical outcomes after IVM were initially suboptimal, with live birth rates per cycle of <20% [29–31]. However, more recent studies in experienced centres have reported improved live birth rates of approximately 35%–40% [32–36]. Nevertheless, the chances of a live birth with IVM are currently still slightly lower than with IVF [35, 36].

Safety of IVM

One of the concerns regarding IVM is the health of babies after utilization of this procedure. Therefore, several studies have investigated the effects of IVM on embryos and children conceived using IVM. Embryos generated from IVM did not show an increase in imprinting [37]. In another study, IVM blastocysts showed similar

TABLE 11.1 Milestones in IVM Development

Year	Development
1969	First experimental IVF with in vitro matured oocytes [9]
1991	First IVM baby resulting from immature oocytes derived from oocyte donors [4]
1994	First babies from IVM oocytes obtained from women with PCOS [15]
1999	First babies from IVM cycles with hCG triggering before oocyte retrieval [16]
2014	First live birth from IVM oocytes after oophorectomy in a patient with ovarian cancer [21]
2020	First report of birth after vitrification of IVM oocytes in a woman with breast cancer [20]
2020	First live births after biphasic IVM in women with PCOS [24]
2020	ESHRE Guideline on Female Fertility Preservation: IVM was regarded as a fertility preservation procedure [22]
2021	Practice Committees of the ASRM: IVM no longer considered an experimental technique; potential for wider clinical application of IVM suggested [3]

Abbreviations: ASRM, American Society of Reproductive Medicine; ESHRE, European Society of Human Reproduction and Embryology; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; PCOS, polycystic ovary syndrome.

rates of methylation and gene expression at germline differentially methylated regions (gDMRs) compared with IVF embryos, and expression of major epigenetic regulators was similar between the IVM and IVF groups [38]. Cellular morphology in IVM oocytes also does not appear to differ from oocytes matured *in vivo* according to transmission electron microscopy [39].

Initial data on the neonatal health and developmental outcome of children conceived using IVM showed no significant differences compared to children born after traditional IVF, with or without intracytoplasmic sperm injection (ICSI) [40–44]. However, the relatively small number of children conceived through IVM compared with IVF limits the accuracy of malformation and anomaly rates, and developmental outcomes cannot yet be adequately assessed.

Recently, a follow-up of babies born to women who participated in a randomized controlled trial comparing new IVM protocol with a pre-maturation step (CAPA IVM) and IVF showed that overall development up to 24 months of age was comparable in children born after CAPA IVM compared with IVF [45]. Another prospective cohort study also showed that the use of CAPA-IVM did not have any significant impact on childhood physical and mental development compared with children born as a result of natural conception, up to 15 months of age [46]. The updated data from the children follow-up studies assure the safety of the new CAPA-IVM technique.

Patient populations for application of IVM

Suggested indications for IVM include patients at risk of OHSS, those with limited time for ovarian stimulation, or where sustained elevations of estradiol are contraindicated [3]. Patient populations particularly suited to the use of IVM are described next.

PCO/PCOS

The fact that ovarian stimulation is not required means that IVM is especially suited to patients with PCOS. This group is at

increased risk of exaggerated ovarian response, including OHSS, ovarian torsion, and thromboembolism associated with high estradiol levels [47–50]. In addition, because the reduced ability of immature oocytes retrieved from mid-antral follicles to resume meiosis and progress to the MII stage is a factor limiting the clinical efficiency of IVM [51], the higher antral follicle count (AFC) in women with PCOS [52, 53] makes them particularly suitable candidates for IVM. This is shown by higher IVM success rates in women with PCOS compared with normo-ovulatory women [54]. Recently, a large randomized controlled trial comparing IVM and IVF in women with a high AFC found that live birth after the first embryo transfer (ET) occurred in 96 women (35.2%) in the IVM group and 118 women (43.2%) in the IVF group (absolute risk difference –8.1%; 95% confidence interval [CI] –16.6%, 0.5%) [35]. Cumulative ongoing pregnancy rates at 12 months after randomization were 44.0% in the IVM group and 62.6% in the IVF group (absolute risk difference –18.7%; 95% CI –27.3%, –10.1%). OHSS did not occur in the IVM group, versus two cases in the IVF group. There were no statistically significant differences between the IVM and IVF groups with respect to the occurrence of pregnancy complications, obstetric and perinatal complications, preterm delivery, birth weight, and neonatal complications [35].

Normo-ovulatory patients

IVM can also be applied in normo-ovulatory women. Initially, clinical pregnancy rates in normo-ovulatory women were 4%–25% [17, 29, 31, 55], but these have improved to approximately 30% [56, 57]. For these women, reasons for choosing to undergo IVM are generally social, and may include financial considerations in countries where the cost of gonadotropins limits access to IVF, and the wish to avoid potential drug-related side effects and the psychological stress associated with IVF [49, 58]. IVM has also been cited as the ART of choice in countries where severe legal restrictions have reduced the success rate of standard IVF protocols [59].

AFC is an important consideration in the selection of suitable candidates for IVM among normo-ovulatory women [51, 58]. A minimum AFC of >5 has been suggested. In addition, it has been suggested that candidates for IVM should have a good prognosis in terms of achieving pregnancy using conventional ART, including the following patient characteristics: age ≤36 years; body mass index <30 kg/m²; FSH levels <10 mIU/mL; estradiol levels <250 pmol/mL; and anti-Müllerian hormone levels >1.3 ng/mL [51, 58].

Fertility preservation

Given that IVM can be done within a relatively short timeframe without the need for ovarian stimulation, it is particularly useful for fertility preservation in patients with cancer who are unable to delay chemotherapy, or in women with breast cancer for whom exposure to elevated estradiol concentrations may accelerate their disease [60, 61]. While IVM is usually conducted during the follicular phase of the menstrual cycle, successful collection and maturation of oocytes has been demonstrated during the luteal phase in patients with cancer, thus making it a good option in those scheduled for imminent chemotherapy [60, 61].

Poor ovarian response

Some patients respond poorly to IVF, including those with low oestrogen levels, few or slow-growing follicles, or smaller follicles

(diameter <12 mm on day 15 of the treatment cycle) [62]. Poor responders to IVF do not appear to benefit from prolonged treatment or higher doses of gonadotropins, and fertilization and pregnancy outcomes may be negatively affected [62, 63]. Thus, alternative ARTs may be useful in this patient group. A case series reported positive outcomes after IVM in eight patients who were poor responders to IVF, with a clinical pregnancy rate of 37.5% [62].

Gonadotrophin-resistant ovary syndrome (GROS)

GROS is a rare endocrine syndrome characterized by hypergonadotropic anovulation (World Health Organization group 3) and consequent infertility. Women with GROS experience amenorrhea (primary or secondary) despite having an intact uterus and vagina, elevated levels of FSH and luteinizing hormone (LH), normal ovarian reserve, no concomitant autoimmune disease, and an inappropriate response to ovarian stimulation [64, 65]. The aetiology of GROS remains unclear. However, both genetics and immunology might play a role. Several mutations with loss of function and single nucleotide morphisms of the FSH receptor (FSHR) have been described to date [66–70]. The lack of response to ovarian stimulation in patients with GROS means that conventional IVF is not possible. Therefore, oocyte donation was previously considered the only option for ART in these patients. However, IVM is increasingly being recognized as a potential alternative [71–73], which would provide the opportunity for a woman with GROS to achieve fertilization of her own oocytes. Several live births after use of IVM in GROS have been reported [64, 72–76].

Rescue of oocytes that have failed to mature in stimulated cycles

Some patients undergoing IVF treatment yield high proportions of immature oocytes after conventional ovarian stimulation [77]. In these patients, IVM is a feasible rescue method for increasing embryo number [77]. A prospective study demonstrated the utility of rescue IVM, concluding that it should become routine practice in women with low functional ovarian reserve [78]. Rescue IVM produced an additional 1.5 embryos for transfer in women with low functional ovarian reserve and 1.6 in patients with normal functional ovarian reserve [78]. Pregnancy and delivery chances were also improved with rescue IVM in women with low functional ovarian reserve [78]. Different time dynamics were noted between women with low functional ovarian reserve and those with normal functional ovarian reserve.

Novel rescue IVM approaches are being developed, including the use of heterologous follicular fluid and supernatant of cumulus-granulosa cells in culture medium to mimic the intact follicular microenvironment [79]. While results appear promising, more data are required to understand the contribution of such approaches to improving clinical outcomes [77].

Unexplained primarily poor-quality embryos

Another reason for IVF failure can be the presence of follicular developmental abnormalities, which results in a lack of available embryos [80]. Management of this group of patients is particularly frustrating because the women appear to respond normally to ovarian stimulation, yet the reasons for very poor-quality embryos are largely unknown [80].

Positive outcomes with IVM have been reported in a study of patients with empty follicle syndrome (i.e. where no oocytes can be retrieved from mature ovarian follicles after controlled

ovarian stimulation despite apparently normal follicular development and estradiol levels) [80]. Of seven patients who had failed to conceive after numerous IVF attempts, two pregnancies were achieved. The authors suggested that oocyte atresia or dysfunctional development in these two particular patients occurred in the more advanced stages of follicular development, and that IVM, with the final maturation occurring *in vitro*, had helped to overcome the processes involved, thus facilitating normal oocyte recovery [80].

Current IVM practices

Generally, IVM has been described as the technique whereby immature oocytes are collected from small follicles, usually <10 mm in diameter. Immature oocytes then are put in an IVM culture system, which allows the immature oocytes to develop and reach the MII stage. Mature oocytes are then inseminated with sperm to produce zygotes and embryos *in vitro*. The additional laboratory component of IVM includes the identification and collection of intact COCs and the IVM culture of intact COCs to obtain mature oocytes for IVF. The insemination technique and embryo culture procedure are similar to that of conventional IVF/ICSI [81].

Retrieved COCs are typically cultured in complex tissue culture-like medium with supplementation of a protein source and hormones for one to two days under atmospheric oxygen. Based on the use (or not) of FSH priming and hCG triggering, and the immature oocyte culture system, current IVM practices can be differentiated into four protocols: standard, hCG priming, biphasic (or CAPA), and rescue (Table 11.2, Figure 11.1) [19]. One of the benefits of a biphasic approach (CAPA IVM) is that most oocytes reach the MII stage (Figure 11.2).

Conventionally, IVM is performed without gonadotropin priming before immature oocyte retrieval. However, early evidence suggested that mild stimulation with FSH (FSH priming) improved both human oocyte yield and maturation competence [17, 18]. Conflicting data from small randomized controlled trials (RCTs) regarding the effectiveness of FSH priming on IVM success have been reported [82, 83]. Recently, a large RCT compared the efficacy of CAPA IVM and conventional IVF/ICSI in women with PCO treated with highly purified hMG 150 IU/day for two days [35]. The results showed that CAPA (biphasic) IVM was non-inferior to IVF in terms of live birth rate after the first ET [35]. Until now, there has been limited evidence regarding the optimal dosage of FSH/hMG used in IVM, but a common dosage is FSH 150 IU/day for two to six days in the follicular phase of the cycle.

The first successful application of hCG triggering before oocyte retrieval in IVM was reported in 1999 [16]. One early study reported that the pregnancy rate might be improved by priming with hCG prior to immature oocyte retrieval [84]. However, later reports provide conflicting results regarding the effectiveness of hCG priming in IVM [85, 86]. In the first decade of the new millennium, use of hCG triggering in IVM was adopted worldwide, mostly combined with FSH priming [87]. The most common approach was to prime with hCG 10,000 IU at 36 hours before the retrieval of immature oocytes. The largest report of IVM using hCG triggering, combined with FSH priming, included 921 women with PCOS [32]. The oocyte maturation rate was 71.2%, implantation rate was 21.5% with cleavage embryos, and the cumulative live birth rate over 12 months after an IVM cycle was 33.7% [32].

TABLE 11.2 Different *In Vitro* Maturation (IVM) Protocols in Clinical Practice

IVM Protocol	Features and Evidence
Standard (Figure 11.1)	<ul style="list-style-type: none"> Conventional IVM protocol With or without FSH priming before oocyte retrieval No hCG trigger After oocyte retrieval, only GV-stage COCs collected All COCs matured to MII oocytes in one phase of IVM culture Promising results [33]
hCG Priming (Figure 11.1)	<ul style="list-style-type: none"> Modified IVM used in many ART centres worldwide hCG trigger before oocyte pickup With or without FSH priming before oocyte retrieval Oocytes retrieved may be at different stages of maturation (MII, MI, or GVBD) MII oocytes inseminated after a few hours, while MI and GVBD oocytes require IVM culture for 24–30 hours Largest report of IVM using hCG triggering and FSH priming ($n = 921$ women with PCOS): oocyte maturation rate 71.2%, implantation rate 21.5% (cleavage embryos), cumulative live birth rate over 12 months after an IVM cycle 33.7% [32]
Biphasic (Figures 11.1 and 11.2)	<ul style="list-style-type: none"> Also known as capacitation (CAPA) IVM Includes a pre-IVM culture and the IVM culture Pre-IVM medium contains C-type natriuretic peptide to block meiotic resumption and maintain oocytes at the GV stage for 24 hours, allowing GV oocytes to gain further cytoplasmic maturation and improved competence prior to IVM culture [88] No hCG trigger FSH priming can be given prior to oocyte retrieval Only GV-stage COCs collected at oocyte retrieval The first clinical reports of CAPA-IVM showed promising results [23, 24]. The CAPA-IVM system significantly improved oocyte maturation, embryo quality, and clinical pregnancy rates versus standard IVM in patients with PCOM. Larger data proved that CAPA-IVM was non-inferior to conventional IVF in terms of live birth rate after the first ET in women with high antral follicle count [35].
Rescue	<ul style="list-style-type: none"> For poor quality, immature GV-stage oocytes collected after conventional ovarian stimulation, including full FSH stimulation and hCG triggering (these have no developmental potential and are usually discarded) IVM performed to achieve mature oocytes (using any protocol) The rationale and efficacy of rescue IVM is still questioned

Abbreviations: ART, assisted reproductive technology; COCs, cumulus–oocyte complexes; FSH, follicle-stimulating hormone; GV, germinal vesicle; GVBD, germinal vesicle breakdown; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; MI, metaphase I; MII, metaphase II; PCOM, polycystic ovarian morphology; PCOS, polycystic ovary syndrome.

Technical issues

Immature oocyte pickup

One of the most important steps that could be challenging for physicians when starting to perform IVM is the immature oocyte pickup (OPU) procedure. Differences between OPU procedures for IVM versus IVF are shown in Table 11.3.

Identify small cumulus–oocyte complex and additional culturing steps

The COCs that are harvested from small follicles are smaller in size and more compacted than those collected from stimulated IVF cycles. An additional filtration step can be applied to harvest very small COCs from follicle fluid. More culturing steps have to be prepared and applied in an IVM treatment cycle for immature oocytes. Embryologists have to obtain new skills in harvesting small COCs and workload is increased in ART laboratories in ART programs practicing IVM.

Fresh or frozen transfer

The traditional approach to ET after IVM was to transfer fresh embryos using hCG administration 36 hours before oocyte pickup (OPU), and endometrial preparation using estradiol and progesterone. This protocol has been used with reasonably good

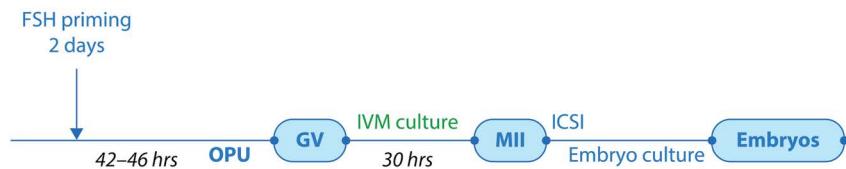
results [32]. However, some studies have reported comparatively low success rates with fresh ET in IVM [36, 89]. It is possible that different IVM methodologies and different endometrial preparation regimens might result in different outcomes. Recently, a randomized, controlled pilot study was designed to compare the effectiveness and safety of a freeze-only strategy and fresh ET in women with a high AFC undergoing IVM with a pre-maturation step (CAPA IVM, without hCG) [90]. The results suggested that use of a freeze-only strategy in patients with a high AFC undergoing IVM with a pre-maturation step could significantly increase the rate of ongoing pregnancy resulting in live birth compared with fresh ET. Other fertility outcomes and complication rates did not differ between the two groups [90], although larger studies with longer follow-up are needed to confirm the comparative safety of frozen versus fresh ET in IVM.

Clinical use of IVM

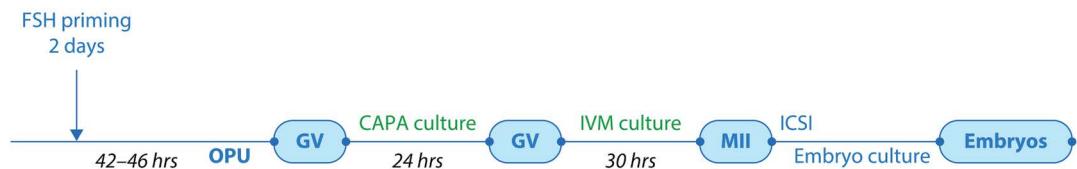
Role of IVM in modern ART

There is still room for further development of more efficient IVM protocols [91]. Recent knowledge about oocyte physiology and development can be translated into clinical practice to improve the efficacy of these protocols.

(a) Standard IVM



(b) Biphasic (CAPA) IVM



(c) hCG Priming IVM

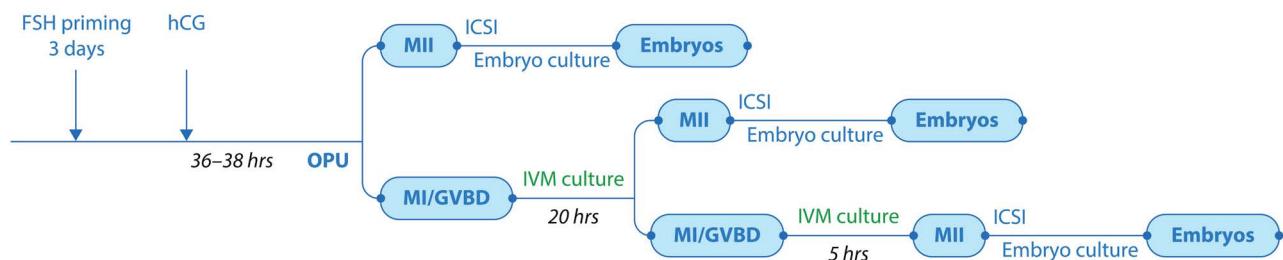


FIGURE 11.1 Current *in vitro* maturation (IVM) protocols, in which oocytes are harvested from small follicles (based on the authors' practice). (a) Standard protocol, without hCG before oocyte pickup (OPU) and one IVM culture step; (b) biphasic (CAPA) protocol, without hCG before OPU and biphasic IVM culture; and (c) hCG priming protocol, with hCG triggering before OPU and collected oocytes at different stages of maturity. Abbreviations: GV, germinal vesicle; ICSI, intracytoplasmic sperm injection; MII, metaphase II.

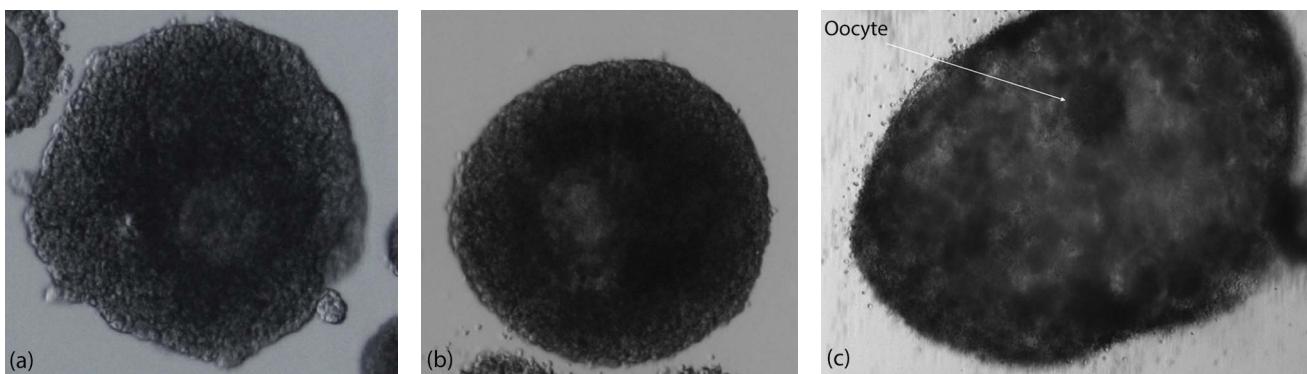


FIGURE 11.2 Cumulus–oocyte complex (COC) in the biphasic (CAPA) *in vitro* maturation (IVM) protocol. (a) COC after oocyte pickup (OPU); oocytes are at the germinal vesicle (GV) stage. (b) COC after CAPA culture; oocytes still at the GV stage. (c) COC after CAPA culture and IVM culture; oocytes reach the metaphase II (MII) stage.

TABLE 11.3 Differences between Oocyte Pickup Procedures for IVM versus IVF

Component	IVM	IVF
Timing	42 hours after FSH injection (standard or biphasic IVM) 36 hours after hCG injection (hCG IVM)	34–36 hours after hCG injection
Ovary characteristics	Ovary: smaller, not vascularized Follicle size: 2–10 mm Stromal tissue: thick, hard	Ovary: large, vascularized Follicle size: ≥14 mm Stromal tissue: soft, small
Needle	Mobile 19–21G Single/Double needle system: outer 17G; inner 19–21G Tubing length: 20–40 cm	Not mobile 17G Single/Double lumen Tubing length: ≥60 cm
Techniques	Aspiration and curetting No flushing	Aspiration ± Flushing
Medium	Heparin added (if necessary)	No heparin added
Duration	20–30 minutes per OPU	10 minutes per OPU

Abbreviations: FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; OPU, oocyte pickup.

IVM is no longer considered an experimental technique. The potential for wider clinical application of IVM was suggested by the Practice Committees of ASRM [3]. However, understanding which groups of patients will benefit from more widespread use of IVM remains a topic of debate. Groups of patients probably best suited for IVM include women at greater risk of adverse events during ovarian stimulation, including OHSS and other potential adverse reactions, and those with cancer who cannot wait for a full ovarian stimulation regime due to the urgency of initiating gonadotoxic cancer treatment or with hormonally sensitive cancer that is a contraindication for standard ovarian stimulation.

One approach is to consider IVM as an alternative ART treatment that is patient-friendlier and can reduce the treatment burden of standard IVF cycles with respect to medication cost, time, and stress relating to the stimulation and follow-up protocols. For young patients undergoing IVF who have a potentially high response to ovarian stimulation and a good prognosis with many extra, unused embryos (that may be cryopreserved or discarded), IVM could be a feasible alternative to IVF with more friendly treatment processes and a lower number of embryos generated, but a good cumulative live pregnancy rate.

Patients with good ovarian reserve but who have low response to ovarian stimulation could also be good candidates for IVM. In addition, IVM is the only option to help women with GROS to have children using their own oocytes [73].

There are several reasons why all major modern ART centres should have IVM facilities and protocols available. These include the likelihood of wider application of IVM in the future, the requirement to manage indications where IVM is the only option (e.g. fertility preservation, GROS), the increasing need for more patient-friendly ART treatment, and the fact that IVM protocols

are improving thanks to advances in knowledge on human follicular and oocyte development.

Barriers to the application of IVM in clinical practice

As mentioned previously, worse outcomes (fewer embryos and lower pregnancy rates, especially cumulative pregnancy rate) with IVM versus IVF [36] have been the major barriers to more widespread implementation of IVM in clinical practice. In addition, there have been concerns about the health of infants born following IVM. High rates of chromosomal abnormalities have been reported in rescue IVM embryos [92, 93], although animal studies have shown a greater risk of imprinting defects in embryos from ovarian stimulation than from IVM [94]. Nevertheless, comparison of IVM with other ARTs (i.e. IVF, ICSI) and/or with spontaneous conception have found no differences in the incidence of congenital anomalies [40, 95, 96].

On a practical level, a lack of standardized protocols for IVM [3], leading to variable outcomes between centres depending on levels of expertise, might also negatively impact the uptake of IVM. Furthermore, IVM may be less attractive to some centres because of the associated increase in laboratory workload, particularly if the hCG-priming protocol is used.

While the costs associated with IVM are lower than those for IVF [2, 27, 28], IVF is covered by health insurance in many Western countries, whereas this may not be the case for IVM, meaning that out-of-pocket costs for patients might be higher. In contrast, for emerging economy countries that often have no reimbursement system for infertility therapies, IVM can be the most effective ART at the lowest out-of-pocket expense for the patient. In this sense, IVM could increase patient access to treatment in lower-income countries in Asia, South America, and Africa.

Finally, the availability of alternative strategies for reducing the risk of OHSS might also negatively impact the use of IVM. However, although other assisted reproductive medicine strategies, such as the use of gonadotropin-releasing hormone agonist trigger and freeze-only cycles, have reduced the rate of OHSS in high-risk women [97–99], cases of severe OHSS have still been reported with these approaches [100, 101]. IVM is the only ART that carries zero risk of OHSS, which is important in regions where critical care units are sparse.

Overcoming barriers to the clinical use of IVM

Strategies to overcome barriers to the use of IVM include specific training for clinicians to aspirate unexpanded COCs from small antral follicles and to adapt alternative therapeutic strategies for the most difficult cases. Specific training for embryologists is mandatory to handle these unexpanded COCs into prolonged pre-maturation “CAPA” systems. Additional research in the field is needed, especially on the health of babies born as a result of IVM. Another important factor is improved recognition of IVM as a women-friendly and efficient ART technique by fertility specialists. Finally, there is a need for increased allocation of funding for IVM research to further develop IVM into the first-line treatment for the aforementioned infertility indications.

References

1. Edwards RG. Maturation *in vitro* of human ovarian oocytes. Lancet. 1965;2:926–29.
2. Paulson RJ, Fauser B, Vuong LTN, Doody K. Can we modify assisted reproductive technology practice to broaden reproductive care access? Fertil Steril. 2016;105:1138–43.

3. Practice Committees of the American Society for Reproductive Medicine, the Society of Reproductive Biologists and Technologists, and the Society for Assisted Reproductive Technology. In vitro maturation: A committee opinion. *Fertil Steril.* 2021;115:298–304.
4. Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril.* 1991;55:109–13.
5. De Vos M, Ortega-Hrepich C, Albuza FK, Guzman L, Polyzos NP, Smitz J, et al. Clinical outcome of non-hCG-primed oocyte in vitro maturation treatment in patients with polycystic ovaries and polycystic ovary syndrome. *Fertil Steril.* 2011;96:860–4.
6. De Vos M, Smitz J, Thompson JG, Gilchrist RB. The definition of IVM is clear-variations need defining. *Hum Reprod.* 2016;31:2411–5.
7. Edwards RG. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature.* 1965;208:349–51.
8. Edwards RG, Donahue RP, Baramki TA, Jones HW Jr. Preliminary attempts to fertilize human oocytes matured in vitro. *Am J Obstet Gynecol.* 1966;96:192–200.
9. Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. *Nature.* 1969;221:632–5.
10. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet.* 1978;2:366.
11. Johnston I, Lopata A, Speirs A, Hoult I, Kellow G, du Plessis Y. In vitro fertilization: the challenge of the eighties. *Fertil Steril.* 1981;36:699–706.
12. Jones HW Jr, Acosta AA, Andrews MC, Garcia JE, Jones GS, Mayer J, et al. Three years of in vitro fertilization at Norfolk. *Fertil Steril.* 1984;42:826–34.
13. Lopata A. Concepts in human in vitro fertilization and embryo transfer. *Fertil Steril.* 1983;40:289–301.
14. Lopata A, Brown JB, Leeton JF, Talbot JM, Wood C. In vitro fertilization of preovulatory oocytes and embryo transfer in infertile patients treated with clomiphene and human chorionic gonadotropin. *Fertil Steril.* 1978;30:27–35.
15. Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertil Steril.* 1994;62:353–62.
16. Chian RC, Gülekli B, Buckett WM, Tan SL. Priming with human chorionic gonadotropin before retrieval of immature oocytes in women with infertility due to the polycystic ovary syndrome. *N Engl J Med.* 1999;341:1624, 1626.
17. Mikkelsen AL, Smith SD, Lindenberg S. In-vitro maturation of human oocytes from regularly menstruating women may be successful without follicle stimulating hormone priming. *Hum Reprod.* 1999;14:1847–51.
18. Wynn P, Picton HM, Krapez JA, Rutherford AJ, Balen AH, Gosden RG. Pretreatment with follicle stimulating hormone promotes the numbers of human oocytes reaching metaphase II by in-vitro maturation. *Hum Reprod.* 1998;13:3132–8.
19. De Vos M, Grynberg M, Ho TM, Yuan Y, Albertini DF, Gilchrist RB. Perspectives on the development and future of oocyte IVM in clinical practice. *J Assist Reprod Genet.* 2021;38:1265–80.
20. Grynberg M, Mayeur Le Bras A, Hesters L, Gallot V, Frydman N. First birth achieved after fertility preservation using vitrification of in vitro matured oocytes in a woman with breast cancer. *Ann Oncol.* 2020;31:541–2.
21. Prasath EB, Chan ML, Wong WH, Lim CJ, Tharmalingam MD, Hendricks M, et al. First pregnancy and live birth resulting from cryopreserved embryos obtained from in vitro matured oocytes after oophorectomy in an ovarian cancer patient. *Hum Reprod.* 2014;29:276–8.
22. Anderson RA, Amant F, Braat D, D'Angelo A, Chuva de Sousa Lopes SM, Demeestere I, et al. ESHRE guideline: Female fertility preservation. *Hum Reprod Open.* 2020;2020:hoaa052.
23. Sanchez F, Le AH, Ho VNA, Romero S, Van Ranst H, De Vos M, et al. Biphasic in vitro maturation (CAPA-IVM) specifically improves the developmental capacity of oocytes from small antral follicles. *J Assist Reprod Genet.* 2019;36:2135–44.
24. Vuong LN, Le AH, Ho VNA, Pham TD, Sanchez F, Romero S, et al. Live births after oocyte in vitro maturation with a prematuration step in women with polycystic ovary syndrome. *J Assist Reprod Genet.* 2020;37:347–57.
25. Das M, Son WY, Buckett W, Tulandi T, Holzer H. In-vitro maturation versus IVF with GnRH antagonist for women with polycystic ovary syndrome: Treatment outcome and rates of ovarian hyperstimulation syndrome. *Reprod Biomed Online.* 2014;29:545–51.
26. Huang JY, Chian RC, Tan SL. Ovarian hyperstimulation syndrome prevention strategies: In vitro maturation. *Semin Reprod Med.* 2010;28:519–31.
27. Ellenbogen A, Shavit T, Shalom-Paz E. IVM results are comparable and may have advantages over standard IVF. *Facts Views Vis Obstyn.* 2014;6:77–80.
28. Gremel AS, Andreadis N, Fatum M, Craig J, Turner K, McVeigh E, et al. In vitro maturation or in vitro fertilization for women with polycystic ovaries? A case-control study of 194 treatment cycles. *Fertil Steril.* 2012;98:355–60.
29. Child TJ, Abdul-Jalil AK, Gulekli B, Tan SL. In vitro maturation and fertilization of oocytes from unstimulated normal ovaries, polycystic ovaries, and women with polycystic ovary syndrome. *Fertil Steril.* 2001;76:936–42.
30. Child TJ, Phillips SJ, Abdul-Jalil AK, Gulekli B, Tan SL. A comparison of in vitro maturation and in vitro fertilization for women with polycystic ovaries. *Obstet Gynecol.* 2002;100:665–70.
31. Söderström-Anttila V, Mäkinen S, Tuuri T, Suikkari AM. Favourable pregnancy results with insemination of in vitro matured oocytes from unstimulated patients. *Hum Reprod.* 2005;20:1534–40.
32. Ho VNA, Pham TD, Le AH, Ho TM, Vuong LN. Live birth rate after human chorionic gonadotropin priming in vitro maturation in women with polycystic ovary syndrome. *J Ovarian Res.* 2018;11:70.
33. Junk SM, Yeap D. Improved implantation and ongoing pregnancy rates after single-embryo transfer with an optimized protocol for in vitro oocyte maturation in women with polycystic ovaries and polycystic ovary syndrome. *Fertil Steril.* 2012;98:888–92.
34. Shalom-Paz E, Holzer H, Son W, Levin I, Tan SL, Almog B. PCOS patients can benefit from in vitro maturation (IVM) of oocytes. *Eur J Obstet Gynecol Reprod Biol.* 2012;165:53–6.
35. Vuong LN, Ho VNA, Ho TM, Dang VQ, Phung TH, Giang NH, et al. In-vitro maturation of oocytes versus conventional IVF in women with infertility and a high antral follicle count: A randomized non-inferiority controlled trial. *Hum Reprod.* 2020;35:2537–47.
36. Walls ML, Hunter T, Ryan JP, Keelan JA, Nathan E, Hart RJ. In vitro maturation as an alternative to standard in vitro fertilization for patients diagnosed with polycystic ovaries: A comparative analysis of fresh, frozen and cumulative cycle outcomes. *Hum Reprod.* 2015;30:88–96.
37. Kuhtz J, Romero S, De Vos M, Smitz J, Haaf T, Anckaert E. Human in vitro oocyte maturation is not associated with increased imprinting error rates at LIT1, SNRPN, PEG3 and GTL2. *Hum Reprod.* 2014;29:1995–2005.
38. Saenz-de-Juano MD, Ivanova E, Romero S, Lolicato F, Sánchez F, Van Ranst H, et al. DNA methylation and mRNA expression of imprinted genes in blastocysts derived from an improved in vitro maturation method for oocytes from small antral follicles in polycystic ovary syndrome patients. *Hum Reprod.* 2019;34:1640–9.
39. Coticchio G, Dal Canto M, Fadini R, Mignini Renzini M, Guglielmo MC, Miglietta S, et al. Ultrastructure of human oocytes after in vitro maturation. *Mol Hum Reprod.* 2016;22:110–8.
40. Buckett WM, Chian RC, Holzer H, Dean N, Usher R, Tan SL. Obstetric outcomes and congenital abnormalities after in vitro maturation, in vitro fertilization, and intracytoplasmic sperm injection. *Obstet Gynecol.* 2007;110:885–91.

41. Mostinckx L, Segers I, Belva F, Buyl R, Santos-Ribeiro S, Blockeel C, et al. Obstetric and neonatal outcome of ART in patients with polycystic ovary syndrome: IVM of oocytes versus controlled ovarian stimulation. *Hum Reprod.* 2019;34:1595–607.
42. Roesner S, von Wolff M, Elsaesser M, Roesner K, Reuner G, Pietz J, et al. Two-year development of children conceived by IVM: A prospective controlled single-blinded study. *Hum Reprod.* 2017;32:1341–50.
43. Söderström-Anttila V, Salokorpi T, Pihlaja M, Serenius-Sirve S, Suikkari AM. Obstetric and perinatal outcome and preliminary results of development of children born after in vitro maturation of oocytes. *Hum Reprod.* 2006;21:1508–13.
44. Yu EJ, Yoon TK, Lee WS, Park EA, Heo JY, Ko YK, et al. Obstetrical, neonatal, and long-term outcomes of children conceived from in vitro matured oocytes. *Fertil Steril.* 2019;112:691–9.
45. Vuong LN, Nguyen MHN, Nguyen NA, Ly TT, Tran VTT, Nguyen NT, Hoang HLT, Le XTH, Pham TD, Smitz JEJ, Mol BW, Norman RJ, Ho TM. Development of children born from IVM versus IVF: 2-year follow-up of a randomized controlled trial. *Hum Reprod.* 2022; 37:1871–9.
46. Nguyen DL, Nguyen NA, Pham TD, Nguyen MHN, Vuong LN. Development of children born after in vitro maturation with a pre-maturation step versus natural conception: A prospective cohort study. *J Assist Reprod Genet.* 2022;39:1959–65.
47. Julania S, Walls MI, Hart R. The place of in vitro maturation in PCO/PCOS. *Int J Endocrinol.* 2018;2018:5750298.
48. Krishnan S, Kaur H, Bali J, Rao K. Ovarian torsion in infertility management - missing the diagnosis means losing the ovary: A high price to pay. *J Hum Reprod Sci.* 2011;4:39–42.
49. MacDougall MJ, Tan SL, Jacobs HS. In-vitro fertilization and the ovarian hyperstimulation syndrome. *Hum Reprod.* 1992;7: 597–600.
50. Ou YC, Kao YL, Lai SL, Kung FT, Huang FJ, Chang SY, et al. Thromboembolism after ovarian stimulation: Successful management of a woman with superior sagittal sinus thrombosis after IVF and embryo transfer: Case report. *Hum Reprod.* 2003;18:2375–81.
51. Fadini R, Mignini Renzini M, Dal Canto M, Epis A, Crippa M, Caliari I, et al. Oocyte in vitro maturation in normo-ovulatory women. *Fertil Steril.* 2013;99:1162–9.
52. Legro RS, Arslanian SA, Ehrmann DA, Hoeger KM, Murad MH, Pasquali R, et al. Diagnosis and treatment of polycystic ovary syndrome: An Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* 2013;98:4565–92.
53. Williams T, Mortada R, Porter S. Diagnosis and treatment of polycystic ovary syndrome. *Am Fam Physician.* 2016;94:106–13.
54. Siristatidis C, Sergentanis TN, Vogiatzi P, Kanavidis P, Chrelias C, Papantoniou N, et al. In vitro maturation in women with vs. without polycystic ovarian syndrome: A systematic review and meta-analysis. *PLoS One.* 2015;10:e0134696.
55. Mikkelsen AL, Smith S, Lindenberg S. Impact of oestradiol and inhibin A concentrations on pregnancy rate in in-vitro oocyte maturation. *Hum Reprod.* 2000;15:1685–90.
56. Fadini R, Colpi E, Mignini Renzini M, Coticchio G, Comi R, Mastrolilli M, et al. Outcome of cycles of oocyte in vitro maturation requiring testicular sperm extraction for nonobstructive azoospermia. *Fertil Steril.* 2011;96:321–3.
57. Fadini R, Dal Canto MB, Mignini Renzini M, Brambillasca F, Comi R, Fumagalli D, et al. Effect of different gonadotrophin priming on IVM of oocytes from women with normal ovaries: A prospective randomized study. *Reprod Biomed Online.* 2009;19:343–51.
58. Lim KS, Chae SJ, Choo CW, Ku YH, Lee HJ, Hur CY, et al. In vitro maturation: Clinical applications. *Clin Exp Reprod Med.* 2013;40:143–7.
59. Dal Canto MB, Mignini Renzini M, Brambillasca F, Cepparo H, Comi R, Villa A, et al. IVM—the first choice for IVF in Italy. *Reprod Biomed Online.* 2006;13:159–65.
60. Maman E, Meirow D, Brengauz M, Raanani H, Dor J, Hourvitz A. Luteal phase oocyte retrieval and in vitro maturation is an optional procedure for urgent fertility preservation. *Fertil Steril.* 2011;95:64–7.
61. Shalom-Paz E, Almog B, Shehata F, Huang J, Holzer H, Chian RC, et al. Fertility preservation for breast-cancer patients using IVM followed by oocyte or embryo vitrification. *Reprod Biomed Online.* 2010;21:566–71.
62. Liu J, Lu G, Qian Y, Mao Y, Ding W. Pregnancies and births achieved from in vitro matured oocytes retrieved from poor responders undergoing stimulation in in vitro fertilization cycles. *Fertil Steril.* 2003;80:447–9.
63. Martin JR, Mahutte NG, Arici A, Sakkas D. Impact of duration and dose of gonadotrophins on IVF outcomes. *Reprod Biomed Online.* 2006;13:645–50.
64. Galvão A, Segers I, Smitz J, Tournaye H, De Vos M. In vitro maturation (IVM) of oocytes in patients with resistant ovary syndrome and in patients with repeated deficient oocyte maturation. *J Assist Reprod Genet.* 2018;35:2161–71.
65. Jones GS, De Moraes-Ruehsen M. A new syndrome of amenorrhea in association with hypergonadotropism and apparently normal ovarian follicular apparatus. *Am J Obstet Gynecol.* 1969;104:597–600.
66. Allen LA, Achermann JC, Pakarinen P, Kotlar TJ, Huhtaniemi IT, Jameson JL, et al. A novel loss of function mutation in exon 10 of the FSH receptor gene causing hypergonadotropic hypogonadism: Clinical and molecular characteristics. *Hum Reprod.* 2003;18:251–6.
67. Bramble MS, Goldstein EH, Lipson A, Ngun T, Eskin A, Gosschalk JE, et al. A novel follicle-stimulating hormone receptor mutation causing primary ovarian failure: A fertility application of whole exome sequencing. *Hum Reprod.* 2016;31:905–14.
68. Katari S, Wood-Trageser MA, Jiang H, Kalyanchuk E, Muzumdar R, Yatsenko SA, et al. Novel inactivating mutation of the FSH receptor in two siblings of Indian origin with premature ovarian failure. *J Clin Endocrinol Metab.* 2015;100:2154–7.
69. Kuehler A, Hauffa BP, Königer A, Kleinau G, Albrecht B, Horsthemke B, et al. An unbalanced translocation unmasks a recessive mutation in the follicle-stimulating hormone receptor (FSHR) gene and causes FSH resistance. *Eur J Hum Genet.* 2010;18:656–61.
70. Nakamura Y, Maekawa R, Yamagata Y, Tamura I, Sugino N. A novel mutation in exon 8 of the follicle-stimulating hormone receptor in a woman with primary amenorrhea. *Gynecol Endocrinol.* 2008;24:708–12.
71. Benamarra A, Fanchin R, Filali-Baba M, Vialard F, Fossard C, Vandame J, et al. Utilization of in vitro maturation in cases with a FSH receptor mutation. *J Assist Reprod Genet.* 2021;38:1311–21.
72. Flageole C, Toufaily C, Bernard DJ, Ates S, Blais V, Chénier S, et al. Successful in vitro maturation of oocytes in a woman with gonadotropin-resistant ovary syndrome associated with a novel combination of FSH receptor gene variants: A case report. *J Assist Reprod Genet.* 2019;36:425–32.
73. Le HL, Ho VNA, Le TTN, Tran VTT, Ma MPQ, Le AH, et al. Live birth after in vitro maturation in women with gonadotropin resistance ovary syndrome: Report of two cases. *J Assist Reprod Genet.* 2021;38:3243–9.
74. Grynberg M, Peltoketo H, Christin-Maitre S, Poulaïn M, Bouchard P, Fanchin R. First birth achieved after in vitro maturation of oocytes from a woman endowed with multiple antral follicles unresponsive to follicle-stimulating hormone. *J Clin Endocrinol Metab.* 2013;98:4493–8.
75. Kornilov NV, Pavlova MN, Yakovlev PP. The live birth in a woman with resistant ovary syndrome after in vitro oocyte maturation and preimplantation genetic testing for aneuploidy. *J Assist Reprod Genet.* 2021;38:1303–9.

76. Li Y, Pan P, Yuan P, Qiu Q, Yang D. Successful live birth in a woman with resistant ovary syndrome following in vitro maturation of oocytes. *J Ovarian Res.* 2016;9:54.
77. Escrich L, Pellicer A, Meseguer M. Let's rescue oocytes: In vitro maturation 2.0 is coming. *Fertil Steril.* 2018;110:638–9.
78. Lee HJ, Barad DH, Kushnir VA, Shohat-Tal A, Lazzaroni-Tealdi E, Wu YG, et al. Rescue in vitro maturation (IVM) of immature oocytes in stimulated cycles in women with low functional ovarian reserve (LFOR). *Endocrine.* 2016;52:165–71.
79. Madkour A, Bouamoud N, Kaarouch I, Louanqli N, Saadani B, Assou S, et al. Follicular fluid and supernatant from cultured cumulus-granulosa cells improve in vitro maturation in patients with polycystic ovarian syndrome. *Fertil Steril.* 2018;110:710–9.
80. Hourvitz A, Maman E, Brengauz M, Machtinger R, Dor J. In vitro maturation for patients with repeated in vitro fertilization failure due to “oocyte maturation abnormalities”. *Fertil Steril.* 2010;94:496–501.
81. Vuong LN, Ho TM, Gilchrist RB, Smitz J. The place of in vitro maturation in assisted reproductive technology. *Fertil Reprod.* 2019;01:11–5.
82. Mikkelsen AL, Høst E, Blaabjerg J, Lindenberg S. Time interval between FSH priming and aspiration of immature human oocytes for in-vitro maturation: A prospective randomized study. *Reprod Biomed Online.* 2003;6:416–20.
83. Mikkelsen AL, Lindenberg S. Benefit of FSH priming of women with PCOS to the in vitro maturation procedure and the outcome: A randomized prospective study. *Reproduction.* 2001;122:587–92.
84. Chian RC, Buckett WM, Tulandi T, Tan SL. Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. *Hum Reprod.* 2000;15:165–70.
85. Reavey J, Vincent K, Child T, Granne IE. Human chorionic gonadotrophin priming for fertility treatment with in vitro maturation. *Cochrane Database Syst Rev.* 2016;11: Cd008720.
86. Sonigo C, Le Conte G, Boubaya M, Ohanyan H, Pressé M, El Hachem H, et al. Priming Before in vitro maturation cycles in cancer patients undergoing urgent fertility preservation: A randomized controlled study. *Reprod Sci.* 2020;27:2247–56.
87. Yang ZY, Chian RC. Development of in vitro maturation techniques for clinical applications. *Fertil Steril.* 2017;108:577–84.
88. Sánchez F, Lolicato F, Romero S, De Vos M, Van Ranst H, Verheyen G, et al. An improved IVM method for cumulus-oocyte complexes from small follicles in polycystic ovary syndrome patients enhances oocyte competence and embryo yield. *Hum Reprod.* 2017;32:2056–68.
89. Ortega-Hrepich C, Stoop D, Guzmán L, Van Landuyt L, Tournaye H, Smitz J, et al. A “freeze-all” embryo strategy after in vitro maturation: A novel approach in women with polycystic ovary syndrome? *Fertil Steril.* 2013;100:1002–7.
90. Vuong LN, Nguyen LK, Le AH, Pham HH, Ho VN, Le HL, et al. Fresh embryo transfer versus freeze-only after in vitro maturation with a pre-maturation step in women with high antral follicle count: A randomized controlled pilot study. *J Assist Reprod Genet.* 2021;38:1293–302.
91. Richani D, Dunning KR, Thompson JG, Gilchrist RB. Metabolic co-dependence of the oocyte and cumulus cells: Essential role in determining oocyte developmental competence. *Hum Reprod Update.* 2021;27:27–47.
92. Nogueira D, Staessen C, Van de Velde H, Van Steirteghem A. Nuclear status and cytogenetics of embryos derived from in vitro-matured oocytes. *Fertil Steril.* 2000;74:295–8.
93. Zhang XY, Ata B, Son WY, Buckett WM, Tan SL, Ao A. Chromosome abnormality rates in human embryos obtained from in-vitro maturation and IVF treatment cycles. *Reprod Biomed Online.* 2010;21:552–9.
94. Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC, Mann MR. Dual effects of superovulation: Loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Hum Mol Genet.* 2010;19:36–51.
95. Chian RC, Xu CL, Huang JY, Ata B. Obstetric outcomes and congenital abnormalities in infants conceived with oocytes matured in vitro. *Facts Views Vis Obgyn.* 2014;6:15–8.
96. Fadini R, Mignini Renzini M, Guarneri T, Dal Canto M, De Ponti E, Sutcliffe A, et al. Comparison of the obstetric and perinatal outcomes of children conceived from in vitro or in vivo matured oocytes in in vitro maturation treatments with births from conventional ICSI cycles. *Hum Reprod.* 2012;27:3601–8.
97. Al-Inany HG, Youssef MA, Aboulghar M, Broekmans F, Sterrenburg M, Smit J, et al. GnRH antagonists are safer than agonists: An update of a Cochrane review. *Hum Reprod Update.* 2011;17:435.
98. Lainas TG, Sfontouris IA, Zorzosilis IZ, Petsas GK, Lainas GT, Alexopoulou E, et al. Flexible GnRH antagonist protocol versus GnRH agonist long protocol in patients with polycystic ovary syndrome treated for IVF: A prospective randomised controlled trial (RCT). *Hum Reprod.* 2010;25:683–9.
99. Tiitinen A, Husa LM, Tulppala M, Simberg N, Seppälä M. The effect of cryopreservation in prevention of ovarian hyperstimulation syndrome. *Br J Obstet Gynaecol.* 1995;102:326–9.
100. Fatemi HM, Popovic-Todorovic B, Humaidan P, Kol S, Bunker M, Devroey P, et al. Severe ovarian hyperstimulation syndrome after gonadotropin-releasing hormone (GnRH) agonist trigger and “freeze-all” approach in GnRH antagonist protocol. *Fertil Steril.* 2014;101:1008–11.
101. Gurbuz AS, Gode F, Ozcimen N, Isik AZ. Gonadotrophin-releasing hormone agonist trigger and freeze-all strategy does not prevent severe ovarian hyperstimulation syndrome: A report of three cases. *Reprod Biomed Online.* 2014;29:541–4.

12

INTRACYTOPLASMIC SPERM INJECTION

Technical Aspects

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Background

The use of assisted reproductive technology (ART) to overcome infertility has increased steadily in the USA and worldwide. Based on 2019 estimates, approximately 448 ART clinics in the USA performed 171,206 ART procedures resulting in 77,998 live deliveries and 83,946 infants [1], contributing to 2.1% of all infants born in the USA, increasing from 2015's estimates of 1.5% [2]. Internationally, the number of non-donor aspirations increased more than 37% from 2010 to 2014 [3] and are projected to continue growing as ART becomes more widely available. Although today's trends indicate a wide acceptance of IVF, it was the development of ICSI that broadened the reaches of ART to serve patients with more severe cases of infertility.

Soon after the establishment of IVF, it became clear that as many as 40% of conventional IVF cycles were affected by fertilization failure or by an extremely low fertilization rate, even though spermatozoa were placed in close proximity to oocytes [4, 5]. This was particularly problematic in patients with diminished sperm motility and/or poor morphology (i.e. it presented a complex obstacle for spermatozoa to penetrate the zona pellucida (ZP), a thick glycoprotein layer surrounding the oocyte [4]). In such cases, gamete micromanipulation was thought to be the only way to overcome this problem. The different techniques developed in this regard focused on assisting the spermatozoon to penetrate the ZP by "softening" it enzymatically with trypsin or pronase [6], penetrating it chemically via localized exposure to acidified Tyrode's solution prior to sperm exposure, or by mechanically piercing the zone pellucida with a microneedle [7]. The placing of the spermatozoon beneath the ZP yielded consistent results, achieving a fertilization rate of ~20% [8]. However, these techniques were abandoned because of limiting factors such as the need for many functional spermatozoa with good progressive motility, and complications like polyspermy [7]. These initial efforts to assist sperm penetration soon became obsolete with the introduction of a microsurgical method for the insertion of spermatozoa directly into the oocyte.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) involves the injection of a single spermatozoon directly into the cytoplasm of an oocyte. ICSI bypasses both the ZP barrier and sperm defects that compromise its ability to fertilize. The ability of ICSI to achieve higher fertilization and pregnancy rates regardless of sperm characteristics makes it the most powerful micromanipulation procedure yet for treating male factor infertility [9]. In fact, the therapeutic possibilities of ICSI range from cases in which, after sperm selection, the spermatozoon show poor progressive motility to its application in azoospermic men where spermatozoa are

micro-surgically retrieved from the epididymis or the testis [10]. While the availability of ART remains variable among European countries given limitations on accessibility and funding, 35 of 43 countries permit treatments to single women and 23 to female couples [11]. ICSI has also made the consistent fertilization of cryopreserved oocytes possible [12], since cryopreservation can lead to physical damage such as premature exocytosis of cortical granules and ZP hardening that inhibit natural sperm penetration [13, 14]. When preimplantation genetic screening is to be performed on oocytes, the removal of the polar body requires the stripping of cumulus corona cells, thus supporting ICSI as the only insemination method to avoid polyspermy. When embryos need to be screened for genetic defects, the avoidance of contaminating spermatozoa on the ZP reduces the chance of unintended amplification by polymerase chain reaction.

While semen processing using a density gradient can remove viral particles from samples from men with HIV, ICSI is the preferred method of insemination by several groups for HIV-discordant couples because it virtually avoids the interaction of oocytes with semen [15–18], requiring fewer attempts to achieve a pregnancy with obviously reduced chances of viral exposure for the unaffected partner and to the conceptus. Reassuringly, no seroconversions have been reported following ART use for HIV-discordant couples [17]. Although it is still unclear whether COVID-19 viral particles are viable in semen [19], initial studies on sperm quality from men with a recent recovery from COVID-19 have shown that semen parameters were significantly lower than the baseline within the first two-month period after hospital discharge [19].

In this chapter, we describe the quintessential technical details involved in the proper execution of ICSI. We also present the clinical outcomes associated with ICSI and appraise its safety.

Ejaculate semen collection and processing

Semen samples are collected by masturbation after two to three days of abstinence and allowed to liquefy for at least 15 minutes at 37°C before analysis. When the semen has high viscosity, this can be reduced within three to five minutes by adding it to 2–3 mL of 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered human tubal fluid (HTF) containing 200–300 IU of chymotrypsin (Sigma Chemical Co., St Louis, MO), which is capable of releasing spermatozoa [20]. Electroejaculation is applied in cases of spinal cord injury or psychogenic anejaculation [21].

Semen concentration and motility are assessed in a Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel). Semen parameters are considered impaired when the sperm concentration is $<16 \times 10^6/\text{mL}$, the total motility is $<42\%$, or normal morphology is exhibited by $<4\%$ of the spermatozoa [22]. Microscopic evaluation on morphology is usually made after

spreading 5 µL of semen or sperm suspension on pre-stained slides (Testsimplets®; Boehringer, Munster, Germany); at least 100–200 spermatozoa are categorized.

For selection, spermatozoa are concentrated by centrifugation at 500 g for five minutes in HTF medium supplemented with 6% (v/v) human serum albumin (HSA; Vitrolife, Englewood, CO). Semen samples with $<5 \times 10^6/\text{mL}$ spermatozoa or <20% total motile spermatozoa are centrifuged in HTF medium at 500–1800 g for five minutes. The resuspended pellet is layered on a discontinuous gradient (ISolate®, Irvine Scientific, Irvine, CA) on two layers (90% and 45%) and centrifuged at 300 g for 10 minutes. A single gradient (90%) is used when samples have a sperm density $<5 \times 10^6/\text{mL}$ spermatozoa and <20% total motile spermatozoa. The densest portion containing the highest concentration of motile spermatozoa of approximately 800 µL is then processed by adding at least 5 mL of HTF medium and centrifuged at 600–800 g for 5–10 minutes to remove the silica gel particles. The concentration of the assessed sperm suspension is adjusted to 1–1.5 $\times 10^6/\text{mL}$ by the addition of HTF medium, and subsequently incubated at 37°C in a gas atmosphere of 5% CO₂ until utilization for ICSI.

Surgical retrieval of spermatozoa

In cases of irreparable obstructive azoospermia, a condition often caused by congenital bilateral absence of the vas deferens and associated with a cystic fibrosis gene mutation, spermatozoa are retrieved by percutaneous or microsurgical epididymal sperm aspiration [23–25]. Variable volumes of fluid (1–500 µL) are collected from the epididymal lumen by a glass micropipette or a metal needle. Since spermatozoa are highly concentrated, only microlitre quantities are needed. When the epididymal approach is not feasible, because of scarring [26], a specimen can be retrieved directly from the testicle by fine needle aspiration [27, 28].

In men with non-obstructive azoospermia (NOA), an open biopsy or, more specifically, a micro-TESE procedure is carried out. Once isolated, seminiferous tubules are mechanically minced in the OR to facilitate the release of spermatozoa [29]. Testicular sperm suspension is then brought to the laboratory and kept in culture for a few hours or overnight until utilization for ICSI.

If spermatozoa are not found, additional biopsies are taken. In these cases, all specimens are brought to the laboratory for further processing by exposure to collagenase type IV (1000 IU/mL) combined with 25 mg/mL of DNase I [26, 30, 31] for one hour, mixing the suspension every 10–15 minutes to enhance enzymatic digestion. Once large portions of undigested tissue, such as tubular walls and connective tissue, are removed, the digested suspension is centrifuged once at 500 g for five minutes or, if needed, at 1500–3000 g for five minutes. Pellets from supernatants and digested tissue are finally resuspended in medium with a final volume ranging from 20 to 200 µL. These samples are allocated into drops covered with oil for extended sperm search in an ICSI dish under an inverted microscope at 400× to assess for presence of germ cells.

Additional testing of the male gamete

Human spermatozoa undergo important modifications in the nuclear chromatin where sperm DNA is supercoiled as protection during transition within the male and female genital tracts.

Shaping of the male gamete nucleus takes place in late spermiogenesis as its chromatin is undergoing a remarkable condensation that renders the sperm DNA transcriptionally inert and highly resistant to the environment. Following the morphological transformation of the nucleus in the seminiferous tubule, as spermatozoa transit through the epididymis, the chromatin is further stabilized through the establishment of disulphide bonds between the thiol-rich protamines [32]. However, DNA damage often remains inevitable in light of these protective measures and can provide insight into the fertilization potential of spermatozoa. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling (TUNEL) and Sperm Chromatin Structural Assay (SCSA; SCSA Diagnostics, Brookings, South Dakota, USA) assess DNA nick-end breaks that occur during mitotic development, but do not distinguish between the types of DNA damage. Similarly, the Comet assay, with its two different forms, the neutral and alkaline, also measures chromatin integrity, the former capable of distinguishing between the double- and single-stranded DNA breaks. On the account of DNA damage, our centre developed an algorithm to best allocate patient treatment plans. Specifically, patients with poor pregnancy outcomes after intrauterine insemination (IUI) were advised to undergo either of the two sperm chromatin fragmentation (SCF) tests. If SCF was within normal limits, patients were advised to proceed to standard *in vitro* insemination; however, patients with elevated SCF were advised to proceed directly to ICSI, and some extreme cases were directed to undergo testicular sperm retrievals [33]. Another test known as Cap-Score™ measures the percentage of spermatozoa undergoing capacitation, the physiological changes this cell must undergo to penetrate and fertilize an egg, by examining ganglioside monosialotetrahexosylganglioside (G_{M1}) localization patterns [34]. Using this assay, patients can minimize the time to achieve pregnancy by proceeding to the most effective ART treatment according to their Cap-Score™.

Several studies have suggested that suboptimal sperm morphology is often associated with aneuploidy, nuclear DNA damage, and, at times, impaired ICSI outcome [35, 36]. It has been postulated that infertile men have compromised DNA integrity as measured by these methods without a correlation with sperm concentration and/or morphology [37]. Conversely, by systematic observations carried out in our laboratory, we have identified an inverse correlation between DNA fragmentation and progressive motility [38]. Perhaps the reason why there is a lack of predictability between DNA integrity and pregnancy outcome with ICSI inseminations may be explained by the fact that only motile spermatozoa are utilized for injection.

The development of sperm sorting devices, such as a commercial microfluidic sperm sorting chip (Zymot Multi device; DxNow, Gaithersburg, MD), which selects spermatozoa based on the progression of their motility, have been shown to isolate sperm with higher genomic competence corroborated by their capacity to generate healthy pregnancies in comparison to sperm selected through standard processing procedures [39–41].

Sperm cryopreservation and thawing

The sperm suspension is supplemented with cryopreservation medium (Freezing Medium-Test Yolk Buffer with Glycerol, Irvine Scientific), and up to 600 µL aliquots of the final solution are placed in cryogenic vials (Nalgene Brand Products, Rochester, NY). The vials are exposed to liquid nitrogen vapor at -70°C for 15 minutes, and then plunged into liquid nitrogen at -196°C.

When needed for ICSI, vials are warmed to room temperature and HTF media is gently added to the thawed suspension and then centrifuged in medium to quickly remove the cryo-protectant. Epididymal spermatozoa and testicular tissue are cryopreserved in a similar manner to the ejaculated counterpart in an excess of cryo-protectant [42–44].

Collection and preparation of oocytes

Baseline bloodwork and pelvic ultrasound are performed on menstrual cycle day 2 for patients treated with gonadotropin-releasing hormone (GnRH) antagonist protocols [45]. Normal baseline parameters include follicle-stimulating hormone (FSH) <12 mIU/mL, oestradiol <75 pg/mL, progesterone <1 ng/mL, and anti-Müllerian hormone (AMH) >1.0 ng/mL. Pelvic ultrasound is performed to evaluate endometrial thickness and to assess the antral follicle count and identification of eventual ovarian cysts.

Controlled ovarian superovulation (COS), human chorionic gonadotropin (hCG) trigger, and oocyte retrieval are performed per standard protocols [45, 46] to maximize follicular response while minimizing the risk of ovarian hyperstimulation syndrome. In general, the hCG trigger is given when at least two lead follicles attain a mean diameter of 17 mm. Oocyte retrieval is performed under conscious sedation using transvaginal ultrasound guidance approximately 35–36 hours after hCG administration. Under the inverted microscope at 100 \times , the cumulus–corona cell complexes are scored as mature, slightly immature, completely immature, or slightly hypermature [47]. Thereafter, the oocytes are ideally incubated for about three and a half hours. Immediately prior to micromanipulation, the cumulus–corona cells are removed by exposure to HTF-HEPES-buffered medium containing 40 IU/mL of Cumulase® (Origio®, Måløv, Denmark). A timely cumulus removal [48, 49] is necessary to observe nucleus maturity and allow holding, visualization, and injection during ICSI. For final removal of the residual corona cells, the oocytes are repeatedly aspirated in and out of a 135–290 μ m micropipette (EZ-Tip®, Research Instruments Ltd, Bickland Industrial Park, UK) mounted on a suction holder (STRIPPER®, ORIGIO, Måløv, Denmark). Each oocyte is then examined under the microscope for overall morphology and whether nuclear maturity, defined as metaphase II (MII) stage characterized by the absence of the germinal vesicle and extrusion of the first polar body, has been reached. ICSI is performed only in oocytes that have reached this level of maturity.

Gamete micromanipulation set-up

The holding pipette (HP-120-30; 120 μ m outer diameter [OD]) and injecting pipette (IC-C1; 5–7 μ m inner diameter [ID]) are both made from glass capillary tubes (Vitrolife AB, Göteborg, Sweden). Both pipettes are bent to an angle of approximately 30° at 1 mm from the tip to manipulate gametes while maintaining the tip of tools horizontally positioned in a plastic Petri dish (ICSI dish, model 351006, Falcon; Becton and Dickinson, Lincoln Park, NJ). Prior to injection, 1 μ L of the sperm suspension is diluted with approximately 8 μ L of a 7% polyvinylpyrrolidone (PVP) solution (90121, Irvine Scientific) and placed in the centre of the ICSI dish. The utilization of a viscous solution helps slow down spermatozoa and allows easier micromanipulation while preventing spermatozoa from sticking to the wall of the injection pipette. Drops 1–8 will contain a single oocyte each. When there are fewer than 100,000/mL spermatozoa in the sample, the sperm

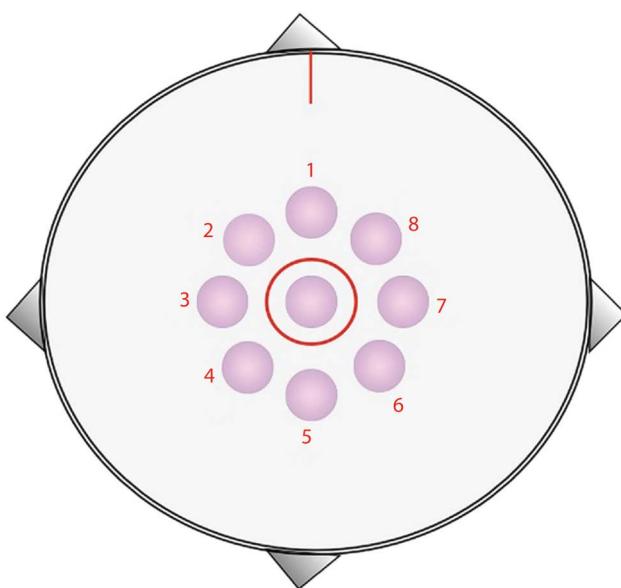


FIGURE 12.1 An intracytoplasmic sperm injection (ICSI) dish is made of 8 μ L drops of ICSI medium plus one central drop overlaid with low-weight paraffin oil. The drops are labelled with a red pencil that is not embryo toxic. The central drop is marked with a circle while the surrounding drops are numbered 1–8 in a counter-clockwise fashion. The central drop is removed and replaced with polyvinylpyrrolidone and spermatozoa, while drops 1–8 will contain a single oocyte each. Specimens with very few spermatozoa are concentrated to a very small volume and placed in drop #8.

suspension is concentrated to approximately 5 μ L and transferred directly in drop #8 (Figure 12.1) while individual oocytes are placed in the remaining drops of G-MOPSTM (Vitrolife) supplemented with HSA (90121, Irvine Scientific). These drops are covered with light-weight paraffin oil (OVOIL, Vitrolife). Following immobilization, an individual spermatozoon is aspirated at the three o'clock edge of the centre drop. For low concentration, a spermatozoon is retrieved from drop #8 and moved to the central drop containing viscous medium to remove debris, gain better aspiration control, and carry out sperm immobilization. The procedure is carried out on a custom designed heated stage (Eastech Laboratory, Centereach, NY) fitted on a Nikon TE2000U inverted microscope at 400 \times using Nikon Modulation contrast optics. The microscope is equipped with a customized micromanipulation set-up (NAI-20P, Narishige Co. Ltd, Tokyo, Japan) consisting of two motor-driven coarse control manipulators and two hydraulic micromanipulators. These custom manipulators have a modified low-position microscope mounting adaptor, a single power supply for the motor-drive coarse unit, and re-routed hydraulic Teflon tubing within the joystick. The micro-tools are controlled by two micro-injectors; one air control (IM-9B) tends to the holding pipette, and the other IM-6 is oil operated and fitted with a metal syringe to finely control pressure in the injection tool.

Selection, immobilization, and loading of the spermatozoon

Although ICSI does not require any specific spermatozoon pre-treatment, gentle immobilization achieved through mechanical pressure is required to concurrently permeabilize the plasma

membrane and outer acrosomal membrane, which allows the release of the sperm cytosolic factor, phospholipase C zeta (PLC ζ). This labile protein triggers a large influx of calcium ions from the endoplasmic reticulum into the cytoplasm, allowing the resumption of oocyte meiosis [50–52]. Qualitative and quantitative modifications of the plasma membrane occurring in the lipidic composition [53] and the absorption of specific proteins, such as beta-defensins and lipocalins, secreted by the epididymal epithelium result in changes of its electric charge and regulation of calcium ion influx of sperm cells [54]. The lack of all these changes is associated with a decreased ability of epididymal spermatozoa to bind and penetrate the oocyte in the case of standard *in vitro* insemination [53]. Owing to physiologic differences in the membrane characteristics of surgically retrieved spermatozoa, a more aggressive immobilization technique is necessary when using epididymal and/or testicular spermatozoa where the sperm tail is rolled over the bottom of the ICSI dish in a location posterior to the mid-piece [55]. This induces a permanent crimp in the flagellum rendering it kinked, looped, or convoluted (Figure 12.2). When aggressive immobilization was applied to surgically retrieved spermatozoa, the more extensive sperm tail disruption prior to oocyte injection appeared to improve the fertilization comparable to one achieved by ejaculated spermatozoa [55–57]. The findings were clarified in a later study where spermatozoa were mechanically immobilized and inserted into the perivitelline space of mouse oocytes [57] to allow ultrathin transmission electron microscopy (TEM) sections. These revealed consistent alterations of sperm plasma membrane including vesiculation, disruption of the acrosomal region, or even loss of the acrosomal content. All of the spermatozoa that were assessed had undergone some membrane disorganization of the head portion, in contrast to the majority of control intact sperm cells. This explains why the immobilization of sperm immediately prior to the ICSI procedure is fundamental for consistent fertilization [55, 57]. An explanation for the enhanced fertilization observed after aggressive immobilization may lie in the structural membrane differences between mature and immature spermatozoa. Immature gametes may require additional manipulation to promote membrane permeabilization, which enhances the post-injection events involved in sperm nuclear decondensation. For example, globozoospermia, a

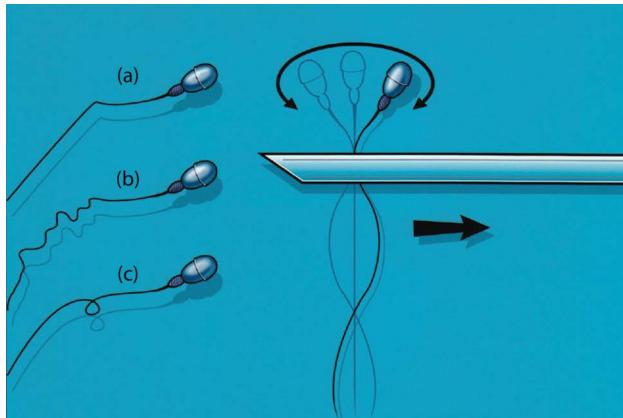


FIGURE 12.2 Aggressive immobilization of the spermatozoon for intracytoplasmic sperm injection. The correctly immobilized spermatozoon has its tail permanently kinked (a), convoluted (b), or looped (c).

condition characterized by round-headed spermatozoa and partial or complete absence of the acrosome, impairs the sperm's ability to bind to the ZP. Although the sperm sample of globozoospermic men may retain normal parameters, they may not be able to fertilize oocytes even through ICSI, thus requiring the addition of an oocyte activating agent to achieve a successful pregnancy [58].

Motile spermatozoa are selected in viscous medium at 400× by observing its shape, light refraction, and motion pattern [59]. After the sperm suspension is loaded in a viscous medium, debris, other cells, bacteria, and immotile spermatozoa remain afloat at the PVP–paraffin oil interface. The viscous environment, by decelerating the spermatozoon, allows evaluation of its tri-dimensional motion pattern, permitting morphological assessment as well as favouring a finely controlled aspiration in the pipette tip. In cases where no motile spermatozoa are seen initially, the eighth drop may be replaced with the sample and supplemented with pentoxifylline to enhance sperm motility. Epididymal spermatozoa and testicular tissue are cryopreserved in the usual manner in an excess of cryo-protectant and spermatozoa are exposed to a motility enhancer (3 mmol/L pentoxifylline) to facilitate the identification of viable spermatozoa [60]. Once a motile spermatozoon is found, it is aspirated and moved into the PVP, where it is immobilized and loaded into the injection pipette for ICSI.

Oocyte injection

The oocyte is held in place by the suction applied to the holding pipette. Prior to piercing the oocyte, the injection pipette is blunted by tapping the tip against the edge of the holding pipette to minimize chances of slashing the oolemma. The inferior pole of the oocyte touching the bottom of the dish allows for better stabilization of the oocyte during the injection procedure. The injection pipette is lowered, and its level is adjusted to reach the focus in accordance with the outer right profile of the oolemma on the equatorial plane at three o'clock. The spermatozoon is then advanced into proximity of the bevelled opening of the injection pipette (Figure 12.3). The pipette is then pushed



FIGURE 12.3 Intracytoplasmic sperm injection procedure. Prior to penetrating the oolemma, the spermatozoon is brought into proximity with the bevelled opening of the injection pipette.

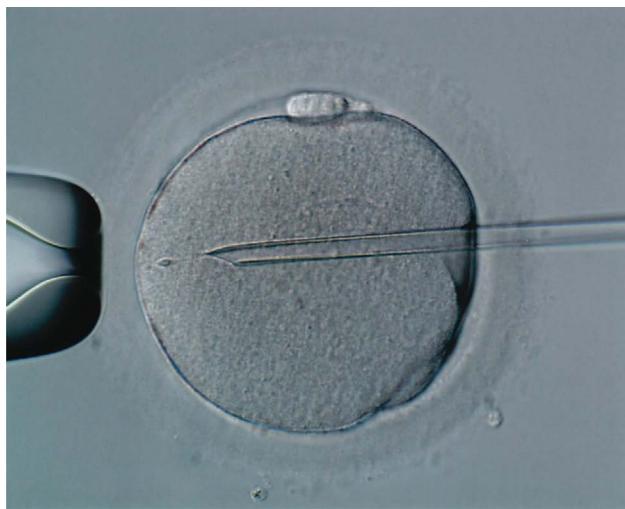


FIGURE 12.4 Intracytoplasmic sperm injection procedure. After the injection pipette has reached the approximate centre of the oocyte, a break in the oolemma is visible as a quivering of the convexities of the membrane above and below the site of penetration.

against the ZP, achieving its penetration through its inner surface to create an invagination of the oolemma. Once the pipette reaches the approximate centre of the oocyte, a breach in the membrane should occur. This is established by a sudden quivering of the convexities (above and below the oolemma invagination), along with a flow motion of the cytoplasmic organelles and the spermatozoon moving upwards into the pipette (Figure 12.4). The spermatozoon is then slowly ejected back into the ooplasm followed by the suction of the cytoplasmic organelle to exert an additional stimulus to trigger the oocyte to resume meiosis. To optimize the interaction with the ooplasm, the sperm cell should be ejected past the tip of the pipette to ensure an intimate position among the ooplasmic organelles that retain the sperm in place while withdrawing the pipette. While the injection pipette is withdrawn, eventual surplus medium is aspirated, resulting in the cytoplasmic organelles tightening around the sperm, and closing the breach generated during injection. Once the pipette is removed, the breach area is observed to make sure the point of entrance maintains a funnel shape with a sealed vertex pointing at the oocyte centre (Figure 12.5).

Evaluation of fertilization and embryo development

After injection, oocytes are loaded into specific culture dish (Embryoslide™, Vitrolife, Englewood, CO), covered in light-weight oil, and placed in an incubator with an integrated time-lapse system (Embryoscope™, Vitrolife, Englewood, CO) [61]. Cultured embryos can be monitored continuously and completely undisturbed while analysing the integrity of the cytoplasm in addition to the number and size of pronuclei. Photos are taken every 12–20 minutes by the device, and thereafter to depict blastomere cleavage, size, and timing in real time. In some cases, 72 hours after microinjection (the afternoon of day 3), those embryos with good morphology are transferred into the uterine cavity. This cleavage stage transfer approach is used particularly in male factor infertility. The number of embryos transferred

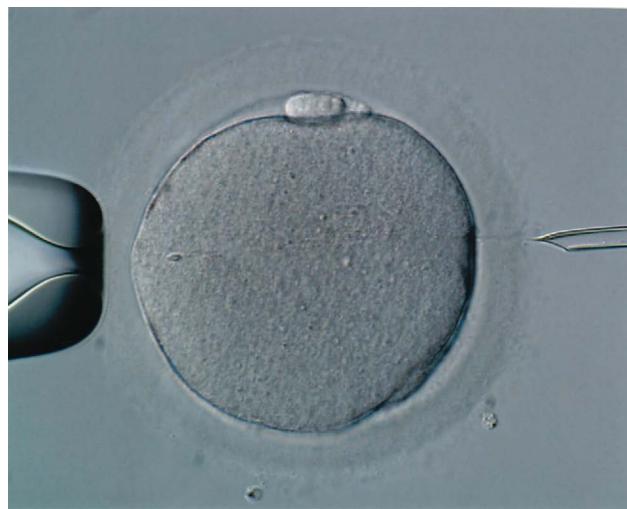


FIGURE 12.5 Intracytoplasmic sperm injection procedure. After the needle is withdrawn from the oocyte, the breach in the oolemma should be observed as a cone-shaped opening with its vertex towards the centre of the oocyte.

depends on embryo availability, quality, and then obviously maternal age.

With the recent development of time-lapse imaging, most embryos are cultured to the blastocyst stage (day 5) to achieve higher implantation potential. The transfer of embryos that completed their full pre-implantation development more closely mirrors the events of natural conception, improving the likelihood that a healthy embryo will implant [62]. This allows for better selection of the best conceptus to maximize pregnancy rates following a single embryo transfer [62, 63].

A comprehensive medium specifically designed for time lapse culture (G-TL, Vitrolife) that support the embryo through its changing physiologic requirements allowing full implantation development [64, 65]. Blastocysts are evaluated according to well-established criteria in order to identify the ideal conceptus to subsequently transfer into the uterine cavity [66–68].

Extended sperm search

When no spermatozoa are identified at the initial semen analysis and even after high-speed centrifugation, an extensive search is carried out. An ICSI dish with the typical PVP solution placed in the central drop is loaded with the pelleted specimen supplemented with pentoxifylline to help augment sperm motility. Each drop is then browsed under 400 \times magnification and eventual spermatozoa identified are picked up and transferred to the PVP drop. The same procedure is performed for surgically retrieved specimens that have been freshly retrieved or recently thawed. Several dishes may have to be made and thoroughly searched for TESE patients until enough spermatozoa are found for injection.

In TESE specimens, sperm may be extremely scarce, requiring, in some cases, an extended sperm search lasting hours to complete depending on the number of oocytes awaiting injection [69]. As expected, the length of time required in acquiring spermatozoa affects clinical outcomes. In general, about 60% of testicular biopsies executed on non-obstructive azoospermic (NOA) men yield injectable spermatozoa (Figure 12.6). At our

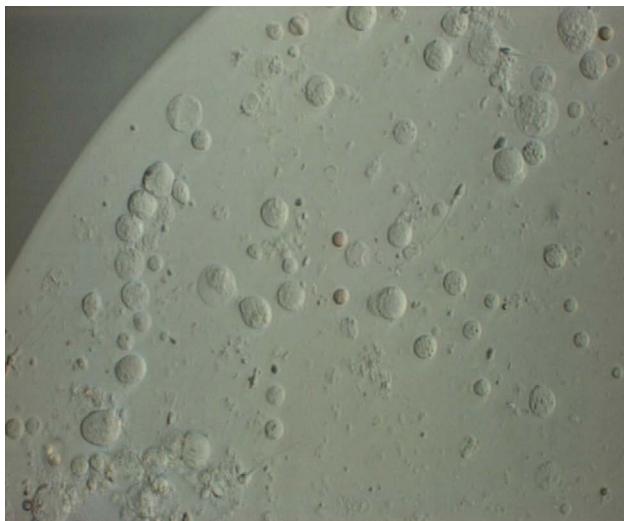


FIGURE 12.6 An example of a testicular sample for an extensive sperm search that has yielded spermatozoa for pickup and injection.

centre, when the time required for extended search time were allotted in groups, requiring from 30 minutes to one hour, one to two hours, two to three hours, and more than three hours, the fertilization rates were 46.5%, 44.9%, 35.5%, and 28.0%, respectively ($P < 0.01$); with the overall clinical pregnancy rates of 41.0%, 34.0%, 31.7%, and 24.2%, respectively ($P < 0.01$); and the overall live birth rates of 35.1%, 26.1%, 21.6%, and 18.4%, respectively ($P < 0.01$) (Figure 12.7). In spite of the fact there was a negative effect on the outcome by increasing search time, the extended search is still important and valuable, as it represents the only opportunity for a man with NOA to bear his own biological child. In fact, even in searches lasting more than three hours, achieving pregnancy is still possible as long as a viable spermatozoon is identified [69].

Clinical results with ICSI

In a cross-sectional survey of ART procedures performed in 76 countries in 2014, the International Committee for Monitoring Assisted Reproductive Technologies reported that 67.4% (619,811 of 919,732) of all cycles utilized ICSI. However, there was considerable variation in ICSI rates, ranging from 53.2% in Asia to 85.5% in Latin America [3]. In examining the incidence of ICSI in the United States, ICSI use increased from 36.4% in 1996 to 76.2% in 2012 and has remained a strong prevalence with 76.6% in 2019 [1, 70]. At our centre, there has been a steady and progressive utilization of ICSI starting at 32.2% in 1993, rising to 48.8% in 1995, reaching 73.6% by 2002, and accounting for more than 90% in 2018 [71, 72].

At our centre, between 1993 and 2021, ICSI has been used in 47,332 cycles compared to 13,737 standard *in vitro* insemination (sIVI) cycles with conventional insemination. ICSI has yielded comparable reproductive outcomes in comparison to conventional sIVI but is also capable of consistently overcoming unforeseen sperm cell dysfunction. The overall fertilization rates after ICSI and sIVI were 73.9% (299,388/404,860) and 60.7% (76,969/126,813), respectively. However, with standard *in vitro* insemination, the fertilization rate is calculated over the total number of oocytes retrieved, so once adopted this denominator for ICSI, the fertilization rate is comparable between the two insemination methods (59.8% ICSI vs. 60.7% IVF). Clinical pregnancy rate, as defined by the presence of a fetal heartbeat on ultrasound, was 39.3% (14,156/36,043) for ICSI compared to 40.2% (4584/11,402) for IVF. Thus far, 21,420 children have been born by the two ART procedures, of which 14,726 were conceived with ICSI. We have performed 47,332 ICSI cycles. Of these, a proportion of 92.2% ($n = 43,643$) of all ICSI cycles were performed using ejaculated spermatozoa, and the remainder involved specimens that were surgically retrieved from the epididymis or testis at our centre. In cycles utilizing ejaculated spermatozoa, a total of 369,662 MII oocytes were injected, resulting in a survival rate of 97.2%. Of those that survived, 80.9% of oocytes fertilized normally, with only one pronucleus and three pronuclei in 2.0% and

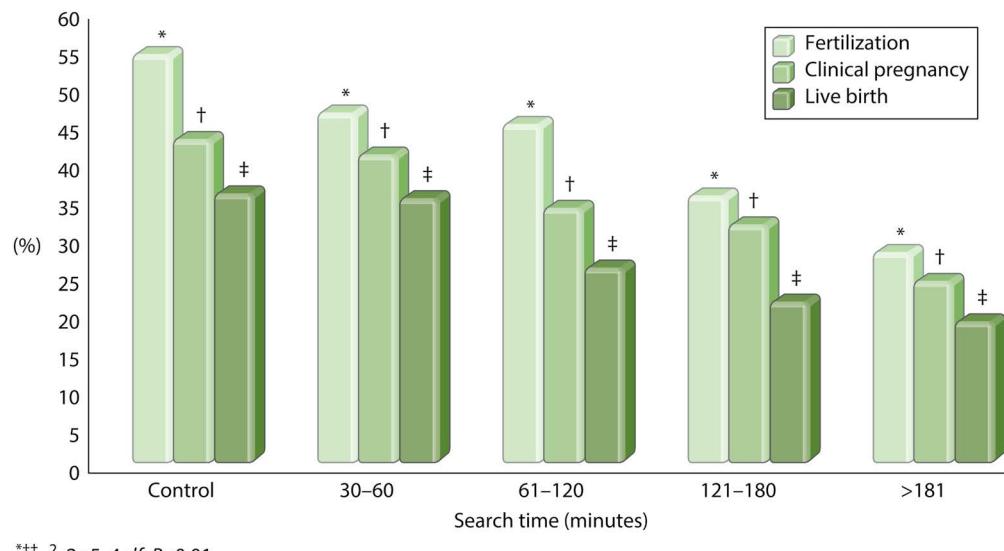


FIGURE 12.7 Pregnancy outcomes according to the length of time spent searching for testicular spermatozoa.

TABLE 12.1 Intracytoplasmic Sperm Injection Outcomes in Men with Severe Oligospermia ($<1 \times 10^6/\text{mL}$ of Spermatozoa)

Parameter	Value
Cycles	3577
Mean concentration (10^6 per mL \pm SD)	0.17 ± 0.25
Mean motility (% \pm SD)	20.5 ± 38.8
Mean morphology (% \pm SD)	0.14 ± 0.52
Fertilization (%)	10,621/17,769 (59.8%)
Clinical pregnancy (%)	1256 (42.8%)

2.9% of oocytes, respectively. No fertilization was noted in 11.1% of oocytes.

Our centre also treats severely oligozoospermic men with a concentration of $<1 \times 10^6/\text{mL}$ of spermatozoa. The outcomes of ICSI cycles in these men are highlighted in Table 12.1. If the initial semen specimen examination showed no spermatozoa, high-speed centrifugation was used.

Table 12.2 summarizes the fertilization and clinical pregnancy rates in ICSI cycles using ejaculated, epididymal, and testicular spermatozoa. When examining the three different sperm sources encompassing all maternal ages, the ejaculated cohort displayed the highest fertilization rates despite encompassing older women ($P < 0.001$). Epididymal spermatozoa achieved a somewhat lower fertilization rate but reported the highest clinical pregnancies as defined by the presence of at least one fetal heartbeat. Cycles using testicular spermatozoa had the lowest fertilization rates overall, despite including the youngest women ($P < 0.001$). The pregnancy rates for patients using testicular spermatozoa were somewhat lower compared to the other

sources. It must be noted that this analysis is purely descriptive, because the surgically retrieved spermatozoa address different clinical indications.

A total of 1295 cycles were performed with epididymal spermatozoa and 1996 cycles were performed with testicular samples. When the fertilization and pregnancy characteristics were analysed according to whether or not the sample was cryopreserved, we observed that after cryopreservation, epididymal samples had lower motility parameters ($P < 0.0001$) and pregnancy outcomes ($P < 0.0001$), though without affecting fertilization rate. When testicular samples were used for ICSI, zygote formation and the ability of the resulting embryo to implant was unaffected (Table 12.3).

The characteristics for 51,457 ICSI cycles including all semen sources are described in Table 12.4.

Of the 21,082 patients presenting with positive βhCG (52.6%), 3244 were biochemical (15.4%) and 869 were blighted ova (4.1%). Among 15,811 patients in whom a viable fetal heartbeat was observed, 1768 had a miscarriage or were therapeutically aborted. The clinical pregnancy rate was 39.4% per embryo transfer (15,811/40,099). A total of 16,843 neonates were born from 14,043 deliveries, including 8376 baby girls and 8467 boys, with an overall frequency of multiple deliveries of 22.8% (3202/14,043): 2989 twins (21.3%), 209 triplets (1.5%), and four quadruplets (0.03%).

Safety of ICSI

At present, 1.5%–6% of children born in developed countries are conceived via ART, where two-thirds of the aspiration cycles included ICSI [3, 73–75]. It is well established that assisted reproduction is associated with adverse perinatal outcomes, including increased risks of preterm delivery, low birth weight, and neonatal mortality [76, 77]. In recent years, there has been considerable

TABLE 12.2 Intracytoplasmic Sperm Injection Outcomes Using Ejaculated, Epididymal, and Testicular Spermatozoa

Parameter	Ejaculated	Epididymal	Testicular
Maternal age (years)	38.4 ± 5	34.2 ± 5	32.8 ± 6
Cycles	43,643	1351	2028
Fertilization rate (%)	238,877/316,691 (75.4)	9317/13,219 (70.5)	10,420/21,242 (49.0)
Clinical pregnancy (%)	18,786 (43.0)	661 (48.9)	807 (39.8)

TABLE 12.3 Semen Parameters and Intracytoplasmic Sperm Injection Outcomes According to Retrieval Sites and Specimen Condition

Outcome	Spermatozoa			
	Epididymal		Testicular	
	Fresh	Frozen/Thawed	Fresh	Frozen/Thawed
Cycles	401	932	1301	800
Density ($10^6/\text{mL} \pm \text{SD}$)	37.1 ± 56	19.2 ± 27	0.44 ± 6	0.22 ± 0.9
Motility (% \pm SD)	19.1 ± 19^a	4.2 ± 10^a	2.4 ± 9^b	0.8 ± 5^b
Fertilization (%)	3058/4269 (71.6)	6114/8673 (70.5)	7171/14585 (49.2)	3790/7652 (49.5)
Clinical pregnancies (%)	223 (60.8) ^c	363 (46.2) ^c	493 (44.7)	239 (39.6)

Notes:

^a Student's t-test, two independent samples, effect of epididymal cryopreservation on sperm motility, $P < 0.0001$.

^b Student's t-test, two independent samples, effect of testicular cryopreservation on sperm motility, $P < 0.0001$.

^c χ^2 , 2 · 2, 1 df, effect of epididymal cryopreservation on clinical pregnancy rate, $P < 0.0001$.

TABLE 12.4 Evolution of Intracytoplasmic Sperm Injection (ICSI) Pregnancies in 51,457 Cycles

No. of	Positive Outcomes		
ICSI cycles	51,457		
Embryo replacements	40,099		
Positive human chorionic gonadotropin	21,082	Pregnancy	52.6% (21,082/40,099)
Biochemical pregnancies	3244		
Blighted ova	869		
Ectopic pregnancies	238		
Positive fetal heartbeats	15,811	Clinical pregnancy	39.4% (15,811/40,099)
Miscarriages/therapeutic abortions	1609		
Deliveries	14,043	Delivery rate	35.0% (14,043/40,099)

work investigating health outcomes in IVF and ICSI children beyond the neonatal period [78, 79]. Follow-ups of children following ART use are highly recommended and needed, however, still onerous [78–80].

ICSI's safety has often been criticized because the fertilizing spermatozoon neither binds to the ZP nor fuses with the oolemma [81]. Bypassing these physiologic steps together with the arbitrary selection of the spermatozoon has been a reason for concern [81–84]. In addition, few studies have been conducted on the effect of male gamete quality on the development of ICSI-conceived children [79]. Thus far, ICSI offspring reaching adolescence and beyond have provided sufficient information to allay these qualms. A study comparing the incidence of congenital abnormalities in ART-conceived and naturally conceived children found no significant difference between the groups [85]. Another series investigating the cognitive development of children born after sIVI or ICSI at ages 3, 5, and 11 found no detrimental effects of their conception on their cognitive abilities when compared with naturally conceived children [86]. In one study evaluating the neurodevelopmental outcomes of two-year-old children born after ART and natural conceptions, the authors found that most two-year-old ICSI children were healthy and were developing normally, as measured by the Bayley Scales of Infant Development [87]. Similarly, no significant difference was observed among the development of two-year-old children when comparing *in vivo* (ovarian stimulation or intrauterine insemination) and *in vitro* (IVF or ICSI) treatments [87]. In a different follow-up of nine-year-old children, it was found that ICSI children and their naturally conceived counterparts had similar behavioural outcomes and IQ scores [88]. Our own centre's evaluation of three-year-old ICSI-conceived children born from fathers with spermatogenic failure displayed no significant delay in developmental skills compared to those ICSI-born children from normozoospermic men [79].

The specific concerns regarding ICSI, whether real or theoretical [89–92], involve the insemination method, the use of spermatozoa with genetic or structural defects, and the possible introduction of foreign genes. Several epidemiological studies of assisted reproduction children report a near twofold increase in the risk of infant malformations [93], a recurrent reduction in birth weight [94], certain rare syndromes related to imprinting errors [95–97], and even a higher frequency of some cancers [98]. However, current evidence does not prove that there is an

increased risk of imprinting disorders and even less so childhood cancers in ICSI children [99, 100]. Epigenetic imbalances have been similarly linked to the exposure of the embryos to long-term culture [101]. To date, Beckwith-Wiedemann, Angelman, Prader-Willi, and Silver-Russell syndromes have been associated with ART procedures [97, 102] and have been found to be equally distributed among the *in vitro* conception methods. At present, there is no evidence that the ICSI insemination itself is responsible for any increase in epigenetic disorders, findings that have been confirmed in animal studies [103].

Considering the thorough investigation into the health and development of children born from ICSI, focus has shifted towards the reproductive health of these individuals. Initially, a study conducted in Belgium found that men born from ICSI were three times more likely to have sperm concentrations below the WHO 2010 reference [104] value of 15 million/mL and four times more likely to have total sperm motility below the WHO reference value of 40% [105]. However, a more recent study offered reassuring data regarding the reproductive capabilities of men conceived through ICSI [106]. Researchers found no significant differences among spermatozoa concentrations from spontaneously conceived men and participants conceived through sIVI or ICSI. Evaluation of serum reproductive hormones from both groups also suggested normal testosterone production, and no correlation was observed between the semen parameters of participants conceived from sIVI or ICSI and their fathers, suggesting those men conceived from ART have comparable reproductive health to their naturally conceived peers [106].

Conclusions

ICSI has established itself as the most reliable technique to overcome fertilization failure via male factor infertility. By pinpointing the beginning of fertilization, it has helped us to better understand important aspects of early gamete interaction. The observed high performance of aggressively immobilized spermatozoa suggests a more efficient destabilization and consequent permeabilization of the sperm membrane, which is responsible for a prompt release of the oocyte-activating factor [107]. These profound physiologic changes induced on the sperm membrane by the action of the injection needle seem to be critically important for immature, surgically retrieved spermatozoa, as confirmed in mammalian studies [55, 108]. It has been demonstrated that the positive outcome of ICSI is largely independent of the basic sperm parameters such as concentration, motility, and morphology. This is particularly evident with cryptozoospermia or when no spermatozoa are present in the ejaculate [59]. It is in these azoospermic men that the surgical isolation of spermatozoa together with ICSI is able to yield fertilization and support embryo development. The possibility of bypassing the steps of testicular and epididymal sperm maturation, acrosome reaction, binding to the ZP, and fusion with the oolemma now permits infertility due to various forms of male factor to be addressed successfully. In fact, in cases of men diagnosed as NOA, as long as a viable spermatozoon is isolated, there is a chance of generating a conceptus. However, we should be cautious about the utilization of ICSI in relation to the acquired evidence that sub-fertile men have a higher frequency of genetic abnormalities that may be passed on through their gametes [109]. Therefore, the earlier concern focused on ICSI insemination itself has shifted to the screening of the sub-fertile man who may transmit his genetic features to the offspring, specifically boys [110, 111]. A large,

worldwide experience suggested that men with extreme male factor conditions caused by a clear genetic make-up such as Klinefelter's syndrome or Yq micro-deletions can be successfully treated by ICSI and still generate healthy offspring [72, 112]. The potential effects of ART on child development should always be kept in mind and the monitoring of child health can be accomplished by a parent-administered questionnaire that provides a cost- and time-effective approach to measuring the child's physical and psychological well-being. In recent years, many studies have provided information on the health of children born after ART, and therefore, current evidence shows that the outcomes of singletons born at term following ART are reassuring [113]. The increased awareness of the risks related to multiple gestations has supported measures aimed at obtaining singleton births, with obvious benefits for the long-term welfare of the offspring.

Despite its success in treating male factor infertility, ICSI does not necessarily achieve the same standard when treating non-male factor infertility. A meta-analysis evaluating clinical outcomes from ICSI in couples with non-male factor showed no advantage to using the procedure to treat non-male factor infertility versus using standard IVF [114]. Similarly, fertilization rates per oocyte retrieved are observed to be lower in ICSI cycles than the standard IVF procedures for these couples as well [115]. In light of the worldwide popularity of ICSI, it is imperative to apply the technique thoughtfully to uphold its fundamental role in infertility treatment [116].

In the evaluation of the infertile male, we still rely on the basic semen analysis measures that can indicate a compromised germ cell maturation process that is altered during spermatogenesis. However, it is still unclear whether semen analysis data provide information on an individual spermatozoon's real potential to generate offspring, rather than reflecting the current status of spermatogenesis. That said, the importance of utilizing additional tests to evaluate spermatogenic characteristics is paramount in providing more insight into the fertilization capacity of sperm [33, 39, 52]. Spermatozoa are not just a vehicle that delivers the male genomic contribution to the oocyte. Upon fertilization, the spermatozoon provides a complete, highly structured, and epigenetically marked genome that, together with a defined complement of RNAs and proteins, plays a distinct role in early embryonic development. Often the origin of male infertility has been associated with specific gene imbalances; although, in many cases, the cause remains idiopathic. Future research will be focused on exploring the effects of genetic variants such as single-nucleotide polymorphisms, copy number variants, differential genome packaging, differential methylation, proteomic changes, and diverse sperm RNAs in order to enlighten the conundrum represented by what we define as male infertility.

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References

- CDC. 2019 Assisted Reproductive Technology Fertility Clinic and National Summary Report. US Department of Health and Human Services, 2021.
- SART. National Summary Report. Society for Assisted Reproductive Technology, 2018.
- Chambers GM, Dyer S, Zegers-Hochschild F, de Mouzon J, Ishihara O, Banker M, et al. International Committee for Monitoring Assisted Reproductive Technologies World Report: Assisted reproductive technology, 2014. *Hum Reprod.* 2021;36(11):2921–34.
- Cohen J, Edwards RG, Fehilly CB, et al. Treatment of male infertility by in vitro fertilization: Factors affecting fertilization and pregnancy. *Acta Eur Fertil.* 1984;15(6):10.
- Rosenwaks Z, Pereira N. The pioneering of intracytoplasmic sperm injection: Historical perspectives. *Reproduction.* 2017;154(6):F71–77.
- Gordon JW, Grunfeld L, Garrisi GJ, Talansky BE, Richards C, Laufer N. Fertilization of human oocytes by sperm from infertile males after zona pellucida drilling. *Fertil Steril.* 1988;50(1):68–73.
- Malter HE, Cohen J. Partial zona dissection of the human oocyte: A nontraumatic method using micromanipulation to assist zona pellucida penetration. *Fertil Steril.* 1989;51(1):139–48.
- Palermo G, Joris H, Devroey P, Van Steirteghem AC. Induction of acrosome reaction in human spermatozoa used for subzonal insemination. *Hum Reprod.* 1992;7(2):248–54.
- Pereira N, Neri QV, Lekovich JP, Spandorfer SD, Palermo GD, Rosenwaks Z. Outcomes of intracytoplasmic sperm injection cycles for complete teratozoospermia: A case-control study using paired sibling oocytes. *Biomed Res Int.* 2015;2015:6.
- Palermo GD, O'Neill CL, Chow S, Cheung S, Parella A, Pereira N. ICSI - State of the Art in Humans. *Reproduction.* 2017;154(6):F93–110.
- Calhaz-Jorge C, De Geyter Ch, Kupka MS, Wyns C, Mocanu E, Motrenko T, et al. Survey on ART and IUI: Legislation, regulation, funding and registries in European countries: The European IVF-monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE). *Hum Reprod Open.* 2020;2020(1):hoz044.
- Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil Steril.* 1997;68(4):724–6.
- Iussig B, Maggiulli R, Fabozzi G, Bertelle S, Vaiarelli A, Cimadomo D, et al. A brief history of oocyte cryopreservation: Arguments and facts. *Acta Obstet Gynecol Scand.* 2019;98(5):550–8.
- Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: Systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update.* 2017;23(2):139–55.
- Zamora MJ, Obradors A, Woodward B, Verneve V, Vassena R. Semen residual viral load and reproductive outcomes in HIV-infected men undergoing ICSI after extended semen preparation. *Reprod Biomed Online.* 2016;32(6):584–90.
- Zafer M, Horvath H, Mmeje O, van der Poel S, Semprini AE, Rutherford G, et al. Effectiveness of semen washing to prevent human immunodeficiency virus (HIV) transmission and assist pregnancy in HIV-discordant couples: A systematic review and meta-analysis. *Fertil Steril.* 2016;105(3):645–55.e2.
- Jindal SK, Rawlins RG, Muller CH, Drobis EZ. Guidelines for risk reduction when handling gametes from infectious patients seeking assisted reproductive technologies. *Reprod Biomed Online.* 2016;33(2):121–30.
- ASRM. Human immunodeficiency virus and infertility treatment: An Ethics Committee opinion. *Fertil Steril.* 2021;115(4):860–9.
- Guo TH, Sang MY, Bai S, Ma H, Wan YY, Jiang XH, et al. Semen parameters in men recovered from COVID-19. *Asian J Androl.* 2021;23(5):479–83.
- Panner Selvam MK, Agarwal A, Sharma R, Samanta L. Treatment of semen samples with α -chymotrypsin alters the expression pattern of sperm functional proteins-a pilot study. *Andrology.* 2018;6(2):345–50.

21. Soeterik TF, Veenboer PW, Oude-Ophuis RJ, Lock TM. Electroejaculation in patients with spinal cord injuries: A 21-year, single-center experience. *Int J Urol.* 2017;24(2):157–61.
22. WHO. WHO Laboratory Manual for the Examination and Processing of Human Semen, 6th ed. Geneva: World Health Organization, 2021.
23. Schlegel PN, Berkeley AS, Goldstein M, Cohen J, Alikani M, Adler A, et al. Epididymal micropuncture with in vitro fertilization and oocyte micromanipulation for the treatment of unrestrictable obstructive azoospermia. *Fertil Steril.* 1994;61(5):895–901.
24. Schlegel PN, Cohen J, Goldstein M, Alikani M, Adler A, Gilbert BR, et al. Cystic fibrosis gene mutations do not affect sperm function during in vitro fertilization with micromanipulation for men with bilateral congenital absence of vas deferens. *Fertil Steril.* 1995;64(2):421–6.
25. McBride JA, Kohn TP, Mazur DJ, Lipshultz LI, Coward RM. Sperm retrieval and intracytoplasmic sperm injection outcomes in men with cystic fibrosis disease versus congenital bilateral absence of the vas deferens. *Asian J Androl.* 2021;23(2):140–5.
26. Friedler S, Raziel A, Strassburger D, Soffer Y, Komarovsky D, Ron-El R. Testicular sperm retrieval by percutaneous fine needle sperm aspiration compared with testicular sperm extraction by open biopsy in men with non-obstructive azoospermia. *Hum Reprod.* 1997;12(7):1488–93.
27. Sheynkin YR, Ye Z, Menendez S, Liotta D, Veeck LL, Schlegel P. Controlled comparison of percutaneous and microsurgical sperm retrieval in men with obstructive azoospermia. *Hum Reprod.* 1998;13(11):3086–9.
28. Vij SC, Sabanegh E. Sperm retrieval from the scrotal extratesticular ductal system. In: Encyclopedia of Reproduction, 2nd ed. Skinner MK (ed.). Oxford: Academic Press, pp. 395–6, 2018.
29. Schlegel PN. Testicular sperm extraction: Microdissection improves sperm yield with minimal tissue excision. *Hum Reprod.* 1999;14(1):131–5.
30. Reis MM, Tsai MC, Schlegel PN, Feliciano M, Raffaelli R, Rosenwaks Z, et al. Xenogeneic transplantation of human spermatogonia. *Zygote.* 2000;8(2):97–105.
31. Cito G, Coccia ME, Sessa F, Cocci A, Verriente P, Picone R, et al. Testicular fine-needle aspiration for sperm retrieval in azoospermia: A small step toward the technical standardization. *World J Mens Health.* 2019;37(1):55–67.
32. Björndahl L, Kvist U. Structure of chromatin in spermatozoa. *Adv Exp Med Biol.* 2014;791:1–11.
33. O'Neill CL, Parrella A, Keating D, Cheung S, Rosenwaks Z, Palermo GD. A treatment algorithm for couples with unexplained infertility based on sperm chromatin assessment. *J Assisted Reprod Genet.* 2018;35(10):1911–7.
34. Schinfeld J, Sharara F, Morris R, Palermo GD, Rosenwaks Z, Seaman E, et al. Cap-score™ prospectively predicts probability of pregnancy. *Mol Reprod Dev.* 2018;85(8–9):654–64.
35. Rodrigo L, Meseguer M, Mateu E, Mercader A, Peinado V, Bori L, et al. Sperm chromosomal abnormalities and their contribution to human embryo aneuploidy. *Biol Reprod.* 2019;101(6):1091–101.
36. Ferrigno A, Ruvolo G, Capra G, Serra N, Bosco L. Correlation between the DNA fragmentation index (DFI) and sperm morphology of infertile patients. *J Assist Reprod Genet.* 2021;38(4):979–86.
37. Agarwal A, Cho CL, Esteves SC. Should we evaluate and treat sperm DNA fragmentation? *Curr Opin Obstet Gynecol.* 2016;28(3):164–71.
38. Palermo GD, Neri QV, Cozzubio T, Rosenwaks Z. Perspectives on the assessment of human sperm chromatin integrity. *Fertility Sterility.* 2014;102(6):1508–17.
39. Parrella A, Keating D, Cheung S, Xie P, Stewart JD, Rosenwaks Z, et al. A treatment approach for couples with disrupted sperm DNA integrity and recurrent ART failure. *J Assisted Reprod Genet.* 2019;36(10):2057–66.
40. Kocur OM, Xie P, Cheung S, Souness S, McKnight M, Rosenwaks Z, Palermo GD. Can a Sperm Selection Technique Improve Embryo Ploidy? *Andrology.* 2022. <https://onlinelibrary.wiley.com/doi/10.1111/andr.13362>.
41. Kocur OM, Xie P, Souness S, Cheung S, Rosenwaks Z, Palermo GD. Assessing male gamete genome integrity to ameliorate poor assisted reproductive technology clinical outcome. *Fertil Steril.* 2023;4:2–10.
42. Wallach EE, Palermo GD, Cohen J, Rosenwaks Z. Intracytoplasmic sperm injection: A powerful tool to overcome fertilization failure. *Fertility and Sterility.* 1996;65(5):899–908.
43. Palermo GD, Cohen J, Rosenwaks Z. Intracytoplasmic sperm injection: A powerful tool to overcome fertilization failure. *Fertil Steril.* 1996;65(5):899–908.
44. Franco JG Jr., Baruffi RL, Mauri AL, Petersen CG, Oliveira JB, Vagnini L. Significance of large nuclear vacuoles in human spermatozoa: Implications for ICSI. *Reprod Biomed Online.* 2008;17(1):42–5.
45. Huang JY, Rosenwaks Z, Kang HJ. How to monitor for best results. In: Cup Book: How to Improve IVF Success Rates, 1st ed. Kovacs G (ed.). Cambridge, UK: Cambridge University Press, pp. 120–6, 2011.
46. Farquhar C, Marjoribanks J, Brown J, Fauser B, Lethaby A, Mourad S, et al. Management of ovarian stimulation for IVF: Narrative review of evidence provided for world health organization guidance. *Reprod Biomed Online.* 2017;35(1):3–16.
47. Eledessy MS, Mehaney AB, Mohamed MA, El Ghadour AEA, Dahi AA. Oocyte score and pregnancy rate in intracytoplasmic sperm injection. *Reprod Health Popul Sci.* 2017;42.
48. Pereira N, Neri QV, Lekovich JP, Palermo GD, Rosenwaks Z. The role of in-vivo and in-vitro maturation time on ooplasmic dysmaturity. *Reprod Biomed Online.* 2016;32(4):401–6.
49. Mizuno S, Ishikawa Y, Matsumoto H, Sato M, Ida M, Fukuda A, et al. The timing of cumulus cell removal for intracytoplasmic sperm injection influences the capability of embryonic development. *Reprod Med Biol.* 2018;18(1):111–7.
50. Dozortsev D, Rybouchkin A, De Sutter P, Qian C, Dhont M. Human oocyte activation following intracytoplasmic injection: The role of The sperm cell. *Hum Reprod.* 1995;10(2):403–7.
51. Palermo G, Joris H, Derde MP, Camus M, Devroey P, Van Steirteghem A. Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Fertil Steril.* 1993;59(4):826–35.
52. Cheung S, Xie P, Parrella A, Keating D, Rosenwaks Z, Palermo GD. Identification and treatment of men with phospholipase C ζ -defective spermatozoa. *Fertil Steril.* 2020;114(3):535–44.
53. Gervasi MG, Visconti PE. Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. *Andrology.* 2017;5(2):204–18.
54. Björkgren I, Sipilä P. The impact of epididymal proteins on sperm function. *Reproduction.* 2019;158(5):R155–r67.
55. Palermo GD, Schlegel PN, Colombero LT, Zaninovic N, Moy F, Rosenwaks Z. Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Hum Reprod.* 1996;11(5):1023–9.
56. Palermo GD, Alikani M, Bertoli M, Colombero LT, Moy F, Cohen J, et al. Oolemma characteristics in relation to survival and fertilization patterns of oocytes treated by intracytoplasmic sperm injection. *Hum Reprod.* 1996;11(1):172–6.
57. Takeuchi T, Colombero LT, Neri QV, Rosenwaks Z, Palermo GD. Does ICSI require acrosomal disruption? An ultrastructural study. *Hum Reprod.* 2004;19(1):114–7.
58. Cheung S, Parrella A, Tavares D, Keating D, Xie P, Rosenwaks Z, et al. Single-center thorough evaluation and targeted treatment of globozoospermic men. *J Assist Reprod Genet.* 2021;38(8):2073–86.
59. Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z. Intracytoplasmic sperm injection: A novel treatment for all forms of male factor infertility. *Fertil Steril.* 1995;63(6):9.

60. Yovich JM, Edirisingshe WR, Cummins JM, Yovich JL. Influence of pentoxifylline in severe male factor infertility. *Fertil Steril*. 1990;53(4):715–22.
61. Kovacs P. Embryo selection: The role of time-lapse monitoring. *Reprod Biol Endocrinol*. 2014;12(1):124.
62. Maheshwari A, Hamilton M, Bhattacharya S. Should we be promoting embryo transfer at blastocyst stage? *Reprod Biomed Online*. 2016;32(2):142–6.
63. Sainte-Rose R, Petit C, Dijols L, Frapsauce C, Guerif F. Extended embryo culture is effective for patients of an advanced maternal age. *Sci Reports*. 2021;11(1):13499.
64. Desai N, Yao M, Richards EG, Goldberg JM. Randomized study of g-TL and global media for blastocyst culture in the EmbryoScope: Morphokinetics, pregnancy, and live births after single-embryo transfer. *Fertil Steril*. 2020;114(6):1207–15.
65. López-Pelayo I, Gutiérrez-Romero JM, Armada AIM, Calero-Ruiz MM, Acevedo-Yagüe PJM. Comparison of two commercial embryo culture media (SAGE-1 step single medium vs. G1-PLUSTM/G2-PLUSTM sequential media): Influence on in vitro fertilization outcomes and human embryo quality. *JBRA Assist Reprod*. 2018;22(2):128–33.
66. Zaninovic N, Nohales M, Zhan Q, de los Santos ZMJ, Sierra J, Rosenwaks Z, et al. A comparison of morphokinetic markers predicting blastocyst formation and implantation potential from two large clinical data sets. *J Assist Reprod Genet*. 2019;36(4):637–46.
67. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertil Steril*. 2000;73(6):1155–8.
68. Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. *Curr Opin Obstet Gynecol*. 1999;11(3):307–11.
69. Palermo GD, Neri QV, Schlegel PN, Rosenwaks Z. Intracytoplasmic sperm injection (ICSI) in extreme cases of male infertility. *PLoS One*. 2014;9(12):e113671.
70. Boulet SL, Mehta A, Kissin DM, Warner L, Kawnass JF, Jamieson DJ. Trends in use of and reproductive outcomes associated with intracytoplasmic sperm injection. *JAMA*. 2015;313(3):255–63.
71. Palermo GD, Neri QV, Rosenwaks Z. To ICSI or not to ICSI. *Semin Reprod Med*. 2015;33(02):92–102.
72. Haddad M, Stewart J, Xie P, Cheung S, Trout A, Keating D, et al. Thoughts on the popularity of ICSI. *J Assist Reprod Genet*. 2021;38(1):101–23.
73. Sunderam S, Kissin DM, Crawford SB, Folger SG, Boulet SL, Warner L, et al. Assisted reproductive technology surveillance – United States, 2015. *MMWR Surveill Summ*. 2018;67(3):1–28.
74. Ferraretti AP, Nygren K, Andersen AN, de Mouzon J, Kupka M, Calhaz-Jorge C, et al. Trends over 15 years in ART in Europe: An Analysis of 6 million cycles. *Hum Reprod Open*. 2017;2017(2):hox012.
75. Berntsen S, Söderström-Anttila V, Wennerholm U-B, Laivuori H, Loft A, Oldereid NB, et al. The health of children conceived by ART: ‘The chicken or the egg?’ *Hum Reprod Update*. 2019;25(2):137–58.
76. Di Tommaso M, Sisti G, Colombi I, Seravalli V, Magro Malosso ER, Vannuccini S, et al. Influence of assisted reproductive technologies on maternal and neonatal outcomes in early preterm deliveries. *J Gynecol Obstet Hum Reprod*. 2019;48(10):845–8.
77. Reig A, Seli E. The association between assisted reproductive technologies and low birth weight. *Curr Opin Obstet Gynecol*. 2019;31(3):183–7.
78. Catford SR, McLachlan RI, O'Bryan MK, Halliday JL. Long-term follow-up of ICSI-conceived offspring compared with spontaneously conceived offspring: A systematic review of health outcomes beyond the neonatal period. *Andrology*. 2018;6(5):635–53.
79. Cheung S, Neri QV, Squires J, Rosenwaks Z, Palermo GD. Assessing the cognitive and behavioral development of 3-year-old children born from fathers with severe male infertility. *Am J Obstet Gynecol*. 2021;224(5):508.e1–e11.
80. Bay B, Lyngsø J, Hohwü L, Kesmodel US. Childhood growth of singletons conceived following in vitro fertilisation or intracytoplasmic sperm injection: A systematic review and meta-analysis. *BJOG*. 2019;126(2):158–66.
81. Pereira N, O'Neill C, Lu V, Rosenwaks Z, Gianpiero DP. The safety of intracytoplasmic sperm injection and long-term outcomes. *Reproduction*. 2017;154(6):F61–70.
82. Rubino P, Viganò P, Luddi A, Piomboni P. The ICSI procedure from past to future: A systematic review of the more controversial aspects. *Hum Reprod Update*. 2015;22(2):194–227.
83. Pereira N, Palermo GD. Ontracytoplasmic sperm injection: History, indications, technique, and safety. In: *Intracytoplasmic Sperm Injection: Indications, Techniques, and Applications*. Palermo GD, Sills ES (eds). Cham, Switzerland: Springer Nature, pp. 9–22, 2018.
84. Palermo GD, Neri QV, Rosenwaks Z. Safety of intracytoplasmic sperm injection. *Methods Mol Biol*. 2014;1154:549–62.
85. Levi Setti PE, Moioli M, Smeraldi A, Cesariello E, Menduni F, Livio S, et al. Obstetric outcome and incidence of congenital anomalies in 2351 IVF/ICSI babies. *J Assist Reprod Genet*. 2016;33(6):711–7.
86. Barbascia A, Mills MC. Cognitive development in children up to age 11 years born after ART—a longitudinal cohort study. *Hum Reprod*. 2017;32(7):1482–8.
87. Balayla J, Sheehy O, Fraser WD, Séguin JR, Trasler J, Monnier P, et al. Neurodevelopmental outcomes after assisted reproductive technologies. *Obstet Gynecol*. 2017;129(2):265–72.
88. Heineman KR, Kuiper DB, Bastide-van Gemert S, Heineman MJ, Hadders-Algra M. Cognitive and behavioural outcome of children born after IVF at age 9 years. *Hum Reprod*. 2019;34(11):2193–200.
89. Bedoschi G, Roque M, Esteves SC. ICSI and male infertility: Consequences to offspring. In: *Male Infertility: Contemporary Clinical Approaches, Andrology, ART and Antioxidants*. Parekattil SJ, Esteves SC, Agarwal A (eds). Cham: Springer International Publishing, pp. 767–75, 2020.
90. Sustar K, Rozen G, Agresta F, Polyakov A. Use of intracytoplasmic sperm injection (ICSI) in normospermic men may result in lower clinical pregnancy and live birth rates. *Aust N Z Obstet Gynaecol*. 2019;59(5):706–11.
91. Avendaño C, Oehninger S. DNA fragmentation in morphologically normal spermatozoa: How much should we be concerned in the ICSI era? *J Androl*. 2011;32(4):356–63.
92. Jiang Z, Wang Y, Lin J, Xu J, Ding G, Huang H. Genetic and epigenetic risks of assisted reproduction. *Best Pract Res Clin Obstet Gynaecol*. 2017;44:90–104.
93. Giorgione V, Parazzini F, Fesslova V, Cipriani S, Candiani M, Inversetti A, et al. Congenital heart defects in IVF/ICSI pregnancy: Systematic review and meta-analysis. *Ultrasound Obstet Gynecol*. 2018;51(1):33–42.
94. Zhu L, Zhang Y, Liu Y, Zhang R, Wu Y, Huang Y, et al. Maternal and live-birth outcomes of pregnancies following assisted reproductive technology: A retrospective cohort study. *Sci Rep*. 2016; 6:35141.
95. Menezo Y, Clément P, Dale B. DNA methylation patterns in the early human embryo and the Epigenetic/Imprinting problems: A plea for a more careful approach to human assisted reproductive technology (ART). *Int J Mol Sci*. 2019;20: 1342.
96. Hattori H, Hiura H, Kitamura A, Miyauchi N, Kobayashi N, Takahashi S, et al. Association of four imprinting disorders and ART. *Clin Epigenetics*. 2019;11(1):21.
97. Cortessis VK, Azadian M, Buxbaum J, Sanogo F, Song AY, Srirrasert I, et al. Comprehensive meta-analysis reveals association between multiple imprinting disorders and conception by assisted reproductive technology. *J Assist Reprod Genet*. 2018;35(6):943–52.
98. Spector LG, Brown MB, Wantman E, Letterie GS, Toner JP, Doody K, et al. Association of in vitro fertilization with childhood cancer in the United States. *JAMA Pediatr*. 2019;173(6):e190392-e.

99. Tang L, Liu Z, Zhang R, Su C, Yang W, Yao Y, et al. Imprinting alterations in sperm may not significantly influence ART outcomes and imprinting patterns in the cord blood of offspring. *PLoS One.* 2017;12(11):e0187869.
100. Spaan M, van den Belt-Dusebout AW, van den Heuvel-Eibrink MM, Hauptmann M, Lambalk CB, Burger CW, et al. Risk of cancer in children and young adults conceived by assisted reproductive technology. *Hum Reprod.* 2019;34(4):740–50.
101. Barberet J, Binquet C, Guilleman M, Doukani A, Choux C, Bruno C, et al. Do assisted reproductive technologies and in vitro embryo culture influence the epigenetic control of imprinted genes and transposable elements in children? *Hum Reprod.* 2021;36(2):479–92.
102. Mussa A, Molinatto C, Cerrato F, Palumbo O, Carella M, Baldassarre G, et al. Assisted reproductive techniques and risk of Beckwith-Wiedemann syndrome. *Pediatrics.* 2017;140(1):e20164311.
103. Wilson TJ, Lacham-Kaplan O, Gould J, Holloway A, Bertoncello I, Hertzog PJ, et al. Comparison of mice born after intracytoplasmic sperm injection with in vitro fertilization and natural mating. *Mol Reprod Dev.* 2007;74(4):512–9.
104. WHO. WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th ed. Geneva: World Health Organization, 2010.
105. Belva F, Bonduelle M, Roelants M, Michielsen D, Van Steirteghem A, Verheyen G, et al. Semen quality of young adult ICSI offspring: The first results. *Hum Reprod.* 2016;31(12):2811–20.
106. Catford SR, Halliday J, Lewis S, O'Bryan MK, Handelsman DJ, Hart RJ, et al. Reproductive function in men conceived with in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril.* 2022;117(4):727–37.
107. Swann K, Lai FA. The sperm phospholipase C-zeta and Ca²⁺ signalling at fertilization in mammals. *Biochem Soc Trans.* 2016;44(1):267–72.
108. Anzalone DA, Iuso D, Czernik M, Ptak G, Loi P. Plasma membrane and acrosome loss before ICSI is required for sheep embryonic development. *J Assist Reprod Genet.* 2016;33(6):757–63.
109. Esteves SC, Zini A, Coward RM, Evenson DP, Gosálvez J, Lewis SEM, et al. Sperm DNA fragmentation testing: Summary evidence and clinical practice recommendations. *Andrologia.* 2021;53(2):e13874.
110. Liu XY, Wang RX, Fu Y, Luo LL, Guo W, Liu RZ. Outcomes of intracytoplasmic sperm injection in oligozoospermic men with Y chromosome AZFb or AZFc microdeletions. *Andrologia.* 2017;49(1):e12602.
111. Golin AP, Yuen W, Flannigan R. The effects of Y chromosome microdeletions on in vitro fertilization outcomes, health abnormalities in offspring and recurrent pregnancy loss. *Transl Androl Urol.* 2021;10(3):1457–66.
112. Palermo GD, Schlegel PN, Sills ES, Veeck LL, Zaninovic N, Menendez S, et al. Births after intracytoplasmic injection of sperm obtained by testicular extraction from men with nonmosaic Klinefelter's syndrome. *N Engl J Med.* 1998;338:588–90.
113. da Silva SG, da Silveira MF, Bertoldi AD, Domingues MR, dos Santos IS. Maternal and child-health outcomes in pregnancies following assisted reproductive technology (ART): A prospective cohort study. *BMC Pregnancy Childbirth.* 2020;20(1):106.
114. Geng T, Cheng L, Ge C, Zhang Y. The effect of ICSI in infertility couples with non-male factor: A systematic review and meta-analysis. *J Assist Reprod Genet.* 2020;37(12):2929–45.
115. Li Z, Wang AY, Bowman M, Hammarberg K, Farquhar C, Johnson L, et al. ICSI does not increase the cumulative live birth rate in non-male factor infertility. *Hum Reprod.* 2018;33(7):1322–30.
116. Keating D, Cheung S, Parrella A, Xie P, Rosenwaks Z, Palermo GD. ICSI from the beginning to where we are today: Are we abusing ICSI? *Global Reproductive Health.* 2019;4(3):e35.

13

HUMAN EMBRYO BIOPSY PROCEDURES

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Introduction

In 1968, Gardner and Edwards reported the possibility of biopsying rabbit embryos with the purpose of sex selection [1]. In this study, rabbit embryos were placed under a dissecting microscope and, using two pipettes and a pair of scissors, some trophectoderm cells were removed from the embryo. The excised cells were then fixed and stained to visualize the Barr Body (i.e. female embryos). Biopsied blastocysts were then transferred to a receiving female rabbit and, once the fetuses were surgically birthed, the sex was confirmed. The first attempt at embryo manipulation on mouse embryos dates back to 1989 [2]. Embryos on day 3 of development were flushed from the female oviduct and one of the eight blastomeres removed mechanically with a pipette. The blastomere was then used for biochemical studies distinguishing between female (XX) and male (XY) embryos by comparing the activity of an X-linked gene with a reference autosomal gene. The first attempt in humans was performed in 1989 when Handyside and colleagues biopsied *in vitro* fertilized embryos at the 6- to 10-cell stage by removing a blastomere through a hole in the zona pellucida (ZP) using a drilling pipette [3]. Due to previous developments in DNA amplification technologies (i.e. PCR), they were able to perform molecular studies where female embryos could be discriminated from male embryos by amplifying target regions on the X chromosome. In 1990, the first pregnancy from a biopsied embryo was reported [4].

Since its first application in the late 1960s, embryo biopsy methodologies have changed radically, not only in the technical approach and the instruments employed for collecting the cellular specimen but also in the developmental stage at which the collection takes place. These changes reflect the continuous improvements that the field of assisted reproduction has undergone over the last decades, including micromanipulation devices and tools, incubation and culture systems, along with the increasing knowledge of embryo physiology generated through clinical experience and research studies. In this chapter, we will discuss the developments in embryo biopsy methods, providing a foundation on the advantages and disadvantages for each approach, and building up a consequential timeline that delineates how pre-implantation genetic testing (PGT) has developed into a robust diagnostic tool for the investigation of embryos' monogenic and chromosomal inheritance.

Embryo biopsy applications

Embryo biopsy is employed in combination with PGT. PGT is mainly employed in cases where the couple in treatment is found or suspected to be at higher risk of having an offspring affected by genetic or chromosomal abnormalities. The analysis of the biopsied cells allows the detection of genetic abnormalities and the subsequent deselection from transfer of the associated embryo.

PGT-A for aneuploidies

An abnormal number of chromosomes (i.e. aneuploidy) is frequently detected in human embryos. This occurrence mainly originates from meiotic errors in the oocyte and therefore affects every cell of the ensuing embryo. The frequency of meiotic errors has been found to be related to women's age and follows a U-shaped curve [5]. Moreover, aneuploidy is the most common genetic abnormality detected in miscarried products of conception [6]. For this reason, PGT-A is mainly employed in patients of advanced maternal age, recurrent pregnancy loss, and repeated implantation failure.

PGT-M for monogenic disorders

Monogenic disorders are caused by a defect in the nucleotide sequence of specific genes. The defective sequence encodes for an altered protein that is unable to carry out its biological function, resulting in abnormal tissue, organ, or systemic physiology, depending on the gene involved (e.g. cystic fibrosis). These defects usually run in the family and are inherited from the parents. Their manifestation mainly depends on their specific type of inheritance (i.e. recessive, dominant, X-linked). However, some monogenic conditions can arise *de novo*, with spontaneous mutations occurring in either the germinal tissue of the parent or in the developing embryo.

PGT-SR for chromosomal structural rearrangements

Chromosome structural rearrangements include inversions and translocations. The first involves the detachment, 180° rotation, and reattachment of a genetic fragment. This event can be harmless if the breaking points are outside of a coding region; however, it can lead to aberrant levels of gene products or dysfunctional genetic regulation if it involves active regions of the chromosome. Translocations involve the detachment of a section of the chromosome and its migration and reattachment to another chromosome. The most typical of these events are Robertsonian translocations where acrocentric chromosomes (i.e. 13, 14, 15, 21, and 22) fuse together. Healthy carriers of a balanced Robertsonian translocation bear almost (if not all) genes without pathological implications. However, their gametes can inherit either normal, balanced, or unbalanced karyotypes. For this reason, they are at higher risk of conceiving a fetus with an abnormal karyotype (and subsequent higher risk of miscarriage).

PGT-HLA for HLA haplotyping

In some countries, couples may access PGT for HLA-typing (i.e. human leukocyte antigen, HLA or major histocompatibility complex, MHC). PGT-HLA allows the selection of an embryo based on its inheritance of specific genes regulating the immune system. This type of embryo selection can be employed to generate a child whose organs are compatible with a sibling affected by a congenital or acquired disorder for whom a hematopoietic stem cell transplantation (HSCT) is needed.

Types of embryo biopsy

Embryo biopsy is the procedure that allows the collection of embryonic cellular material and its DNA to subsequently use as substrate for genetic analysis. The information gathered from the biopsy specimen is used to infer the chromosomal and genetic composition of the whole embryo. Embryo biopsy can be performed at different developmental stages of the embryo or the gametes (Figure 13.1). These include polar body (PB) (hence on the mature oocyte prior to injection on day 0 and/or at the zygote stage on day 1 post insemination), cleavage (on day 3), and blastocyst stage biopsy (on day 5, 6, or 7). According to the latest report from the ESHRE PGT Consortium, which reflects clinical data collected between 2016 and 2017, the embryo developmental stage at which the biopsy is carried out varies according to the downstream PGT analysis [7]. Cleavage stage biopsy is employed in over 75% of PGT-M and 65% of PGT-SR cases, whilst the remaining involve blastocyst biopsy. On the other hand, blastocyst biopsy was carried out in over 85% of PGT-A cases and over 90% of instances where PGT-A was performed in combination with PGT-M or PGT-SR. Each approach has its advantages and limitations mainly revolving on (i) quantity of DNA material harvested (which impacts the robustness and reproducibility of the genetic analysis), (ii) representativeness of the biopsy specimen of the whole embryo's genetic status, (iii) impact of the procedure on embryo viability, and (iv) operational efficiency of the procedure (e.g. percentage of detected healthy embryos over number of biopsy procedures carried out).

Despite the developmental stage at which the procedure is carried out, this consists of three steps: (i) opening of the ZP, (ii) removal/collection of the specimen (either PB(s), blastomeres, or trophectoderm cells), and (iii) tubing of the specimen (or cell fixation on a glass support in case of fluorescence in situ hybridization or FISH analysis).

Zona pellucida opening

ZP opening can be carried out using three different approaches: mechanical, chemical, or through laser [8]. The mechanical method was the first employed and it involved the breaking of the ZP using a sharp microneedle operated through the micromanipulator [9]. This application is nowadays very rare due to access to more advanced techniques (i.e. laser). The chemical approach entails a topical dissolution of the ZP using Tyrode's acid [10]. This was achieved by loading the chemical agent into a microneedle and its subsequent release near the ZP area where access was required for biopsy. At its introduction, this technique was widely used during cleavage stage biopsy, as the acid was very effective at dissolving the ZP. However, the possible harmfulness of the acid to the embryo has led many IVF laboratories to move away from this

approach, in favour of laser. The laser approach is currently the most used method for opening the ZP. It involves the use of a medical grade laser beam to quickly and accurately create a hole with a user-defined diameter in the ZP [11]. Several randomized trials reported no differences among the three different approaches for zona breaching [12]. However, the laser-assisted method is currently the most used due to its standardization, reproducibility, and time effectiveness. Nevertheless, irrespective of the method used, ZP opening can have itself a negative effect on embryo development. One study demonstrated that ZP drilling can compromise the subsequent hatching process and lead to the development of blastocysts with smaller diameter and thicker ZP [13].

Polar body biopsy

PB biopsy was developed as a pre-implantation diagnostic approach alternative to cleavage stage biopsy. Genetic defects are the main cause of spontaneous abortions during the first trimester of pregnancy, with chromosomal abnormalities responsible for about 65% to 70% of them [14]. Most of the aneuploidies found in embryos and fetuses are of maternal origin [15, 16]. This phenomenon is due to a progressive damage of the meiotic apparatus in the oocyte that is developmentally suspended in dictyotene (prophase of meiosis I). When ovulation occurs and maturation (i.e. meiosis) is resumed, the impairments accrued by the meiotic molecular machinery can cause abnormal chromosomal segregation, resulting in aneuploidy [17]. The degree of damage to this apparatus is dependent on female age, and its consequences are confirmed by the drastic increase in aneuploidy rate in patients of advanced maternal age. Being the by-product of female meiosis, polar bodies reflect the content of the oocyte. Therefore, their analysis has the advantage of predicting the maternal contribution to the embryonic genotype, without interfering with the embryo itself. Furthermore, PB biopsy is the only pre-implantation diagnostic alternative that can be offered to couples living in countries where embryo biopsy (post-fertilization) is not allowed.

PB biopsy can be performed using two strategies: (i) simultaneous and (ii) sequential biopsy. In the simultaneous PB biopsies, the two polar bodies (PB1 and PB2) are collected at the same time between six and nine hours after insemination [18, 19]. On the other hand, the sequential approach consists in the biopsy of the two polar bodies at different time points: PB1 is removed one hour after oocyte retrieval and PB2 after fertilization assessment (16–18 hours post-insemination). The opening of the ZP is performed either with a laser or mechanically. In case of sequential biopsy, a second opening in the ZP may be necessary, but it should be avoided whenever possible as it may have a negative effect on blastocyst hatching. After biopsy, the oocytes or zygotes can be cryopreserved or transferred back to the culture dish for

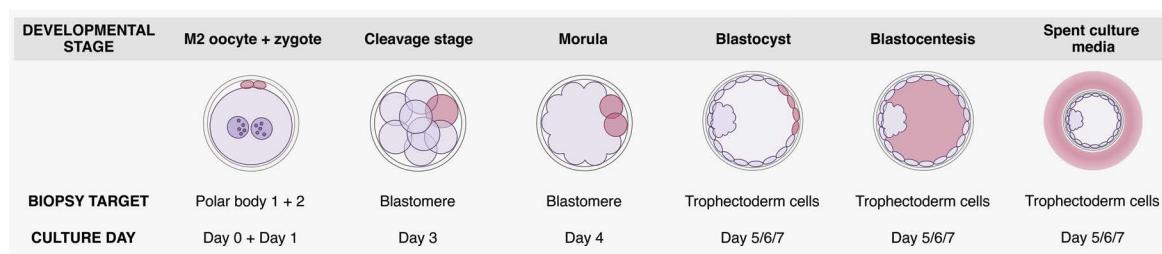


FIGURE 13.1 Developmental stages at which biopsy and embryonic DNA collection can be performed. Cellular (i.e. polar bodies, blastomere, and trophectoderm cells) and extracellular (i.e. blastocoel and spent culture media) targets are shown in pink.

further *in vitro* development. Beside the advantages mentioned previously, genetic investigations based on PB biopsy are limited to the maternal genome, whereas the paternal genomic contribution and the potential mitotic errors occurring in the first stages of embryonic development are completely neglected.

Furthermore, PB biopsy is very time-consuming and not cost-effective, as biopsies need to be performed at inconvenient hours, and two samples per embryo must be collected and analysed regardless of their further development. In a reference study highlighting the limitations of this type of biopsy, the diagnostic accuracy was also questioned. The concordance between karyotype results from PB biopsy and blastocyst biopsy was as low as 60% [20]. This lack of concordance between methodologies may be due to the more comprehensive analysis provided by the blastocyst biopsy approach, which considers not only the maternal contribution but also paternal meiotic errors and mitotic segregation errors occurring during embryonic development. These intrinsic biological and technical limitations make PB biopsy of limited value in the diagnostic field. In fact, due to this evidence, PB biopsy applications are infrequent and limited to specific situations.

Cleavage-stage biopsy

Cleavage embryo biopsy or blastomere biopsy is performed on day 3 post-insemination, when the embryo displays between 6 and 14 cells, before it reaches the compaction stage. The genetic constitution of the whole embryo is inferred from the genetic content of the cell biopsied. In certain circumstances, two blastomeres have been collected to either improve genetic testing accuracy (i.e. by doubling the amount of starting DNA) and/or to minimize the risk of no diagnosis in case the first biopsied cell had lysed. Nevertheless, one cell biopsy is strongly recommended, as the removal of two cells involves a depletion of about 25% of the embryonic mass, with negative effects on the clinical outcome [21]. The timing of cleavage biopsy can correspond with the beginning of the blastomeres compaction process, which starts at around 8 cells and completes around 32 cells, prior to cavitation. This phenomenon makes detachment and isolation of individual blastomeres difficult. For this reason, embryos are commonly exposed to $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free media for 5–10 minutes prior to biopsy. The absence of these salts in the culture solution results in the loosening of cell adhesion, allowing for easier manipulation. However, embryo exposure to this environment should be minimized to avoid major impact on cytoskeleton stability and intracellular communication processes [22]. During cleavage-stage biopsy procedures, the blastomere can be removed mainly by using two approaches: aspiration and extrusion. Both of these methods require the zona to be opened through, as mentioned earlier, Tyrode's acid, mechanical piercing, or, more commonly, using laser pulse. The aspiration method involves making contact between the pipette and the target blastomere through the ZP opening, followed by gentle aspiration of the cell until a good hold is established. After that, the blastomere is carefully pulled out of the ZP. This is the most common method for cleavage-stage biopsy. The extrusion method entails the application of mechanical pressure with a blunt pipette against the ZP, without direct contact with the cell, aiming to dislodge the target blastomere from the remaining embryo through the ZP opening.

After blastomere removal, cell nucleus should be visualized prior to tubing. Biopsied blastomere can then be fixed on a microscope glass support for FISH or tubed for DNA amplification.

There is growing evidence that even at this very early stage of development, a portion of embryonic cells have lost totipotency

and are already partially committed into a specific cell lineage [23, 24]. Moreover, the spatial organization and points of contact between cells may be crucial for correct embryonic development. Compaction reversal and blastomere removal may therefore impact the ability of the embryo to follow specific architectural organization and hinder the formation of tissues required for further development and implantation.

Clear evidence about detrimental effects of cleavage-stage biopsy on embryo reproductive competence was provided by several studies. Scott et al. compared the implantation rate of top-quality sibling embryos, where one of them was submitted to cleavage-stage biopsy and the other was used as a control [25]. Day 3 blastomere biopsy was significantly associated with reduced implantation rate, with only 30% of biopsied embryos resulting in post-transfer sustained implantation, compared to 50% of the unbiopsied embryos.

The usefulness and efficacy of PGT based on blastomere biopsy are also affected by issues associated with the analysis of single cell DNA, impacting both technical (e.g. low amount of DNA results in higher chance of allele drop out (ADO), preferential amplification (PA), chimerical DNA molecules formation, and amplification failure), and biological aspects (single cell results in reduced representativeness of embryo's genetic constitution, e.g. mosaicism). Indeed, mosaicism (i.e. the coexistence of two or more karyotypes within the same embryo) has been detected at higher rates at the cleavage stage, compared to other stages of pre-implantation development [26]. In conclusion, despite the vast experience in its use worldwide, cleavage-stage biopsy appears to be gradually replaced by safer and more robust approaches like trophectoderm biopsy, especially in cases where multicellular specimens provide higher diagnostic robustness (i.e. PGT-A).

Morula biopsy

The human embryo reaches the morula stage between day 3 and day 5 post-insemination, when approximately 16–32 blastomeres are highly compacted. Similar to the procedure used for cleavage-stage biopsy, the embryo must be exposed to $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium to loosen intercellular junctions, thus allowing cell removal. Compared to cleavage-stage biopsy, morula-stage biopsy allows the procurement of more than one cell, thus improving overall diagnostic robustness and reliability. Nonetheless, this approach is rarely used due to the necessity of thoroughly reversing the compaction process, with possible downstream effects on embryo physiology and developmental ability.

Blastocyst biopsy

Following the morula stage, the cavitation process takes place, resulting in the formation of the blastocoel, a fluid filled cavity surrounded by trophectoderm cells (TE). As the blastocyst expands and the blastocoel increases in volume, the inner cell mass (ICM) becomes visible, protruding towards the centre of the cavity. ICM and TE are the first two distinguishable cell lineages of the developing embryo. While TE cells differentiate into extraembryonic tissues (e.g. placenta, chorion), ICM cells form the "embryo proper" from which eventually derive every cell, tissue, and organ of the ensuing fetus. In 2004, the TE biopsy approach was first described clinically by de Boer et al. [27], while the first live births following the procedure were reported in 2005 [28, 29]. Due to the more advanced developmental phase of the embryo, blastocyst-stage biopsy allows the collection of multiple cells (i.e. between 5 and 10) without interfering with the ICM (Box 13.1). Blastocyst biopsy is usually performed between

BOX 13.1 TECHNICAL INFORMATION ON TROPHECTODERM BIOPSY

LABORATORY REQUISITES

- Laboratory experienced and proficient in extended embryo culture
- Adequate equipment including:
 - Incubators in accordance with the workload
 - Micromanipulation station equipped with laser system

Equipment

- Laminar airflow cabinet
- Inverted microscope with micromanipulation system and heated stage
- Medical grade Laser (usually integrated in a x40 objective)
- Cooling rack
- Thermostat/incubator
- Incubator for embryo culture
- Vitrification system
- -20°C freezer

N.B. All devices should be calibrated, and their performance routinely validated.

Materials

- Capillaries for standard manipulation (i.e. 140 µm for biopsy specimen, 240 µm for vitrification, 300 µm for blastocyst transfer)
- Micromanipulation dishes (e.g. ICSI dishes)
- Sterile PCR tubes (i.e. 0.2 mL)
- Pre-warmed culture oil (i.e. 37°C)
- Holding micropipette (conventionally at 35° angle)
- Biopsy micropipette (conventionally at 35° angle, 10–15 µm diameter for PB biopsy; 30–35 µm diameter for cleavage-stage biopsy; 25–30 µm diameter for blastocyst biopsy)

Reagents

- Pre-warmed HEPES or MOPS buffered medium
- Biopsy sample washing solution
- Biopsy sample loading solution
- PVP for micropipette priming (optional)
- Surface decontaminant effective on DNA and DNase

Biopsy procedure

Pre-set-up

- Decontaminate surfaces, instruments, and work areas with appropriate, non-embryo toxic solutions (e.g. Oosafe)
- Wear protective clothing to minimize chance of sample DNA contamination (e.g. face mask, powder-free gloves)
- Embryologist must work in accordance with validated protocols
- Ensure all the material required is available, sterile, and within the expiration date
- Ensure correct traceability through labelling of dishes and tubes

Set-up

- Plate out dishes using pre-warmed (37°C) HEPES or MOPS-buffered media
- Overlay with pre-warmed (37°C) culture oil
- Place in thermostat (non-gassed) to equilibrate
- Set up micromanipulator with holding and biopsy pipettes
- Calibrate laser

Procedure

- When TE cells are herniating, transfer the embryo to biopsy dish avoiding blastocyst collapse.
- Orientate the embryo to have the ICM close to the side of the holding pipette and the herniating cells on the side of the biopsy pipette.

(Continued)

- Make minor adjustments to the embryos orientation to have the point of herniation on the same plane as the holding pipette.
- Make contact with the herniating cells gently aspirating them inside the biopsy pipette.
- Through combined action of suction and traction, acquire 5–10 cells within the pipette and expose cell junctions outside of the ZP.
- Apply a couple of laser beams to the cell junctions while applying traction on the aspirated cells until detached. Alternatively, apply “flicking” method by releasing the blastocyst from the holding pipette and move its lower edge against the upper edge of the biopsy pipette. Maintaining both pipettes in focus, swiftly move the biopsy pipette up, excising the target cells using the friction between the pipettes.

Tubing

- Prepare biopsy wash dishes immediately before use at room temperature, aliquoting 20 µL drops.
- Transfer the biopsied cells to the first drop and rinse them through serial wash drops.
- Transfer the specimen into the sterile, prelabelled PCR tube in 1 µL volume.
- To minimize DNA degradation, the PCR tube containing the specimen should be kept in a cooling rack until transfer to a –20°C freezer.

CONTINUOUS PERFORMANCE MONITORING

In order to achieve consistent outcomes, TE biopsy should be performed only by trained operators [56]. To avoid service disruption and work overload, more than one qualified operator should be available in the clinic. Operators' performance should be periodically monitored to ensure consistency of service. Main parameters to be tracked include:

- Rate of conclusive (aneuploid/euploid) and inconclusive diagnosis (amplification failure/non-concurrent result)
- Embryo degeneration rate after biopsy and embryo survival rate after warming
- Pregnancy rate and live birth rate per transfer

day 5 and day 7 post-insemination, depending on the embryo's development rate, expansion stage, and presence/identifiability of the ICM. There are three main protocols in the literature that describe how to perform a TE biopsy (Figure 13.2). The first method was published by McArthur and colleagues in 2005, then Capalbo et al. published two other methods in 2014 and 2016 [29–31].

The major difference among these protocols is when ZP opening is performed.

The protocol described by McArthur et al. entails a laser-assisted ZP opening at the cleavage stage, followed by extended culture up to the blastocyst stage [29]. ZP opening on days 3 to 4 post-insemination facilitates TE cells herniation, which, under pressure from the enlarging blastocoel, extrude from the ZP, making their biopsy easier. This approach is commonly used and shows both advantages and limitations. The main benefit of this method involves an easier ZP opening procedure, as the space between blastomeres and ZP is larger and therefore the laser (or acid) is less likely to affect the embryo. However, the procedure exposes the embryo to (i) suboptimal culture conditions for longer, as it needs to be removed from the incubator twice (i.e. for ZP opening and biopsy); and (ii) the risk of ICM herniation through the ZP opening, which makes the biopsy procedure more challenging.

The method presented by Capalbo et al. in 2014 avoids manipulation of the embryo on day 3 [30]. On the other hand, it requires simultaneous ZP opening and TE cells excision on the day of full blastocyst expansion. Blastocyst hatching is indeed left to occur spontaneously. This approach allows the embryologist to select the portion of TE to biopsy, rather than having to target the cells extruded through the ZP opening. Once the ICM is clearly visible, the blastocyst is anchored to the holding pipette orienting

the ICM between 7 and 11 o'clock. This approach avoids direct anchoring of the ICM by the holding pipette, while keeping it as far as possible from the biopsy area.

The second protocol described by Capalbo et al. in 2016 suggests a ZP opening using a laser early on the day of blastocyst formation, followed by further incubation [31]. This approach promotes and expedites TE herniation in the following hours. Following TE cells extrusion cells, the herniating cells are collected using a combination of gentle suction from the biopsy pipette and laser beam pulses directed at cell junctions to separate the target cells from the rest of the embryo. Similar to McArthur's strategy, this approach requires the embryo to be manipulated over two sessions; however, ICM herniation is avoided. Moreover, compared to the first method described by Capalbo, ZP opening may speed up logistics as cell herniation is facilitated compared to spontaneous hatching.

In the study discussed earlier, Scott et al. also assessed the impact of blastocyst stage biopsy on embryo's reproductive potential [25]. Differently from embryos biopsied at the cleavage stage, embryos that underwent TE biopsy had comparable implantation rates to non-biopsied sibling embryos. The reason for the higher performance of blastocyst stage compared to cleavage stage biopsy are mainly three: (i) TE is a non-embryonic tissue and the risk of interfering with cells committed to ICM differentiation is avoided; (ii) the biopsy is performed after embryonic genome activation, hence the embryo has higher reproductive potential compared to an embryo at an earlier developmental stage; and (iii) a smaller proportion of embryonic mass is removed (5–10 cells out of around 100). Furthermore, blastocyst stage biopsy ensures a more accurate and robust diagnosis. Specifically, all validation studies to date reported a 98%–100% correct prediction of meiotic errors through comprehensive chromosomal

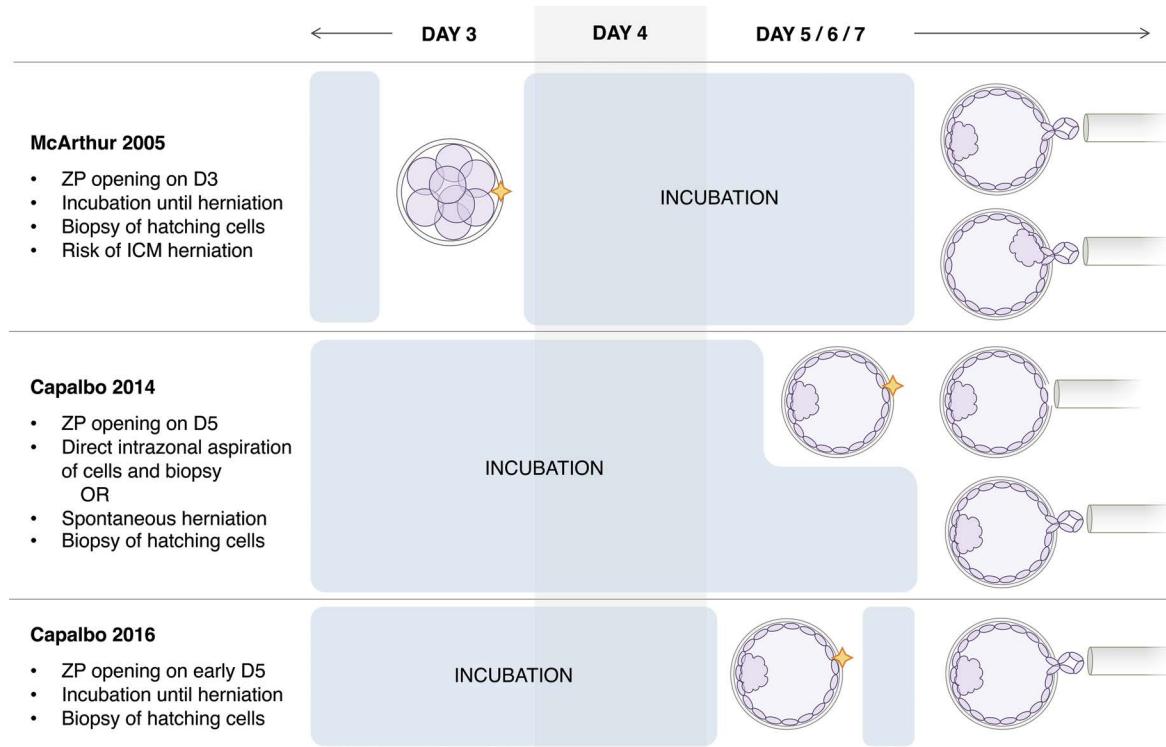


FIGURE 13.2 Three different strategies for zona opening and trophectoderm biopsy. McArthur 2004 involves ZP opening on day 3 of culture and subsequent biopsy of herniating cells at the blastocyst stage (day 5/6/7). Limitations of this approach include potential herniation of ICM cells and double exposure of the embryo to suboptimal conditions (i.e. outside of the incubator). Capalbo 2014 involves ZP opening on day 5 and immediate biopsy of TE cells by contacting the cells within the intra-zonal space. This method avoids both double exposure of the embryo and ICM herniation; however, it may be more technically challenging for less experienced operators. Capalbo 2016 involves ZP opening on day 5 followed by short incubation to allow herniation of TE cells. This method requires double exposure of the embryo to suboptimal conditions; however, it avoids ICM herniation.

screening (CGT), a significant improvement from previous cytogenetic approaches (i.e. FISH) [32, 33]. The collection of multicellular biopsies, combined with sensitive next-generation sequencing technologies also allow the detection of intermediate chromosome copy numbers, possibly associated with mosaicism. However, technical accuracy in detecting mosaicism and its impact on embryo's health and reproductive competence are still at the centre of current scientific debate [34].

Non-invasive biopsy

Although there is no evidence of detrimental effects on the embryos when the biopsy procedure is carried out by experienced professionals, the learning curve for biopsy is steep. As more IVF laboratories look to implement PGT procedures, they face expensive and time-consuming training for their laboratory personnel. For this reason, several groups have investigated and developed non-invasive strategies for collection of embryo-derived DNA which could be employed as alternatives to embryo biopsy. To date, two non-invasive methods have been proposed, both relying on the collection of cell-free embryonic DNA: blastocoel fluid (BF) aspiration (i.e. blastocentesis) and spent culture media.

Blastocentesis

Blastocentesis consists in the aspiration of the BF from the embryonic cavity using a minimally invasive procedure employing an ICSI needle inserted through the TE wall [35–37]. During

the aspiration of the fluid, which is enriched of fragmented DNA of embryonic origin, aspiration of cellular material should be avoided as excluded, free-floating cellular fragments may not be representative of the rest of the embryo. Once the sample has been obtained, it is then tubed for any genetic-molecular analysis. However, blastocoel-derived DNA has been shown to be present in low quantity and poor quality [38]. Accordingly, a number of studies have reported varying efficiencies in DNA amplification, ranging between 34.8% and 87.5% [37, 39–42]. Furthermore, significant differences affect concordance rates at single chromosome level between blastocentesis and conventional biopsy procedures [35, 40, 43–45].

Despite its easier application compared to TE biopsy, this technique requires further development and validation before clinical application can be considered.

Spent culture media

Embryonic DNA can also be found in the culture media where the embryo is being cultured in (i.e. spent culture media, SCM). Different studies have reported the presence of embryonic DNA in the SCM as early as day 2 or 3 of development [46]. This approach is completely non-invasive as it is based on the collection of cell-free DNA accumulated in the culture media and it does not involve embryo manipulation [47, 48]. Although DNA quantity and integrity appear to be superior in SCM compared

to the BF, overall parameters are inferior to TE biopsy specimens [49]. Nonetheless, cell-free DNA availability is dependent on both length of embryo culture prior to SCM collection and external DNA contamination [50]. It has been shown that culture media change on day 4 of culture, followed by SCM collection on day 5/6/7 can increase DNA yield and reduce maternal DNA carry-over from either cumulus cells or in cell-free state [51]. However, although improvements in DNA amplification rate (i.e. >95%) have been obtained by several groups [51–53], the overall diagnostic efficiency of this approach significantly varies across settings and remains unsatisfactory compared to TE biopsy approach even in the best scenarios (e.g. ~80% [50, 51, 54, 55]). Nevertheless, the extremely attractive prospect of obtaining a genetic diagnosis for an embryo without having to biopsy any of its cells surely warrants further endeavours aimed at improving the diagnostic output of this strategy.

References

- Edwards RG, Gardner RL. Sexing of live rabbit blastocysts. *Nature*. 1967;214(5088):576–7.
- Wilton LJ, Shaw JM, Trounson AO. Successful single-cell biopsy and cryopreservation of preimplantation mouse embryos. *Fertil Steril*. 1989;51(3):513–7.
- Handyside AH, Pattinson JK, Penketh RJ, Delhanty JD, Winston RM, Tuddenham EG. Biopsy of human preimplantation embryos and sexing by DNA amplification. *Lancet Lond Engl*. 1989;1(8634):347–9.
- Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*. 1990;344(6268):768–70.
- Yan J, Qin Y, Zhao H, Sun Y, Gong F, Li R, et al. Live birth with or without preimplantation genetic testing for aneuploidy. *N Engl J Med*. 2021;385(22):2047–58.
- Hassold T, Hunt P. To err (meiotically) is human: The genesis of human aneuploidy. *Nat Rev Genet*. 2001;2(4):280–91.
- van Montfoort A, Carvalho F, Coonen E, Kokkali G, Moutou C, Rubio C, et al. ESHRE PGT Consortium data collection XIX–XX: PGT analyses from 2016 to 2017. *Hum Reprod Open*. 2021;2021(3):hoab024.
- ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group, Kokkali G, Coticchio G, Bronet F, Celebi C, Cimadomo D, et al. ESHRE PGT Consortium and SIG Embryology good practice recommendations for polar body and embryo biopsy for PGT. *Hum Reprod Open*. 2020;2020(3):hoa020.
- Cohen J, Malter H, Fehilly C, Wright G, Elsner C, Kort H, et al. Implantation of embryos after partial opening of oocyte zona pellucida to facilitate sperm penetration. *Lancet Lond Engl*. 1988;2(8603):162.
- Cohen J. Assisted hatching of human embryos. *J In Vitro Fert Embryo Transf*. 1991;8(4):179–90.
- Feichtinger W, Strohmer H, Fuhrberg P, Radivojevic K, Antinori S, Pepe G, et al. Photoablation of oocyte zona pellucida by erbium-YAG laser for in-vitro fertilisation in severe male infertility. *Lancet Lond Engl*. 1992;339(8796):811.
- Cimadomo D, Capalbo A, Ubaldi FM, Scarica C, Palagiano A, Canipari R, et al. The impact of biopsy on human embryo developmental potential during preimplantation genetic diagnosis. *BioMed Res Int*. 2016;2016:7193075.
- Kirkegaard K, Juhl Hindkjaer J, Ingerslev HJ. Human embryonic development after blastomere removal: A time-lapse analysis. *Hum Reprod*. 2012;27(1):97–105.
- Menasha J, Levy B, Hirschhorn K, Kardon NB. Incidence and spectrum of chromosome abnormalities in spontaneous abortions: New insights from a 12-year study. *Genet Med Off J Am Coll Med Genet*. 2005;7(4):251–63.
- Hassold T, Hall H, Hunt P. The origin of human aneuploidy: Where we have been, where we are going. *Hum Mol Genet*. 2007;16(R2):R203–8.
- Rabinowitz M, Ryan A, Gemelos G, Hill M, Baner J, Cinnioglu C, et al. Origins and rates of aneuploidy in human blastomeres. *Fertil Steril*. 2012;97(2):395–401.
- Zielinska AP, Bellou E, Sharma N, Frombach AS, Seres KB, Gruhn JR, et al. Meiotic kinetochores fragment into multiple lobes upon cohesin loss in aging eggs. *Curr Biol*. 2019;29(22):3749–3765.e7.
- Strom CM, Ginsberg N, Rechitsky S, Cieslak J, Ivakhenko V, Wolf G, et al. Three births after preimplantation genetic diagnosis for cystic fibrosis with sequential first and second polar body analysis. *Am J Obstet Gynecol*. 1998;178(6):1298–306.
- Montag M, Köster M, Strowitzki T, Toth B. Polar body biopsy. *Fertil Steril*. 2013;100(3):603–7.
- Capalbo A, Bono S, Spizzichino L, Biricik A, Baldi M, Colamaria S, et al. Reply: Questions about the accuracy of polar body analysis for preimplantation genetic screening. *Hum Reprod*. 2013;28(6):1733–6.
- Cohen J, Wells D, Munné S. Removal of 2 cells from cleavage stage embryos is likely to reduce the efficacy of chromosomal tests that are used to enhance implantation rates. *Fertil Steril*. 2007;87(3):496–503.
- Sefton M, Johnson MH, Clayton L, McConnell JML. Experimental manipulations of compaction and their effects on the phosphorylation of uvomorulin. *Mol Reprod Dev*. 1996;44(1):77–87.
- Condic ML. Totipotency: What it is and what it is not. *Stem Cells Dev*. 2014;23(8):796–812.
- Boiani M, Casser E, Fuellen G, Christians ES. Totipotency continuity from zygote to early blastomeres: A model under revision. *Reproduction*. 2019;158(2):R49–65.
- Scott RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: A randomized and paired clinical trial. *Fertil Steril*. 2013;100(3):624–30.
- Li X, Hao Y, Elshewy N, Zhu X, Zhang Z, Zhou P. The mechanisms and clinical application of mosaicism in preimplantation embryos. *J Assist Reprod Genet*. 2020;37(3):497–508.
- de Boer KA, Catt JW, Jansen RPS, Leigh D, McArthur S. Moving to blastocyst biopsy for preimplantation genetic diagnosis and single embryo transfer at Sydney IVF. *Fertil Steril*. 2004;82(2):295–8.
- Kokkali G, Vretou C, Traeger-Synodinos J, Jones GM, Cram DS, Stavrou D, et al. Birth of a healthy infant following trophectoderm biopsy from blastocysts for PGD of beta-thalassaemia major. *Hum Reprod Oxf Engl*. 2005;20(7):1855–9.
- McArthur SJ, Leigh D, Marshall JT, de Boer KA, Jansen RPS. Pregnancies and live births after trophectoderm biopsy and pre-implantation genetic testing of human blastocysts. *Fertil Steril*. 2005;84(6):1628–36.
- Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, et al. Correlation between standard blastocyst morphology, euploidy and implantation: An observational study in two centers involving 956 screened blastocysts. *Hum Reprod Oxf Engl*. 2014;29(6):1173–81.
- Capalbo A, Romanelli V, Cimadomo D, Girardi L, Stoppa M, Dovere L, et al. Implementing PGD/PGD-a in IVF clinics: Considerations for the best laboratory approach and management. *J Assist Reprod Genet*. 2016;33(10):1279–86.
- Fragouli E, Alfarawati S, Daphnis DD, Goodall NN, Mania A, Griffiths T, et al. Cytogenetic analysis of human blastocysts with the use of FISH, CGH and aCGH: Scientific data and technical evaluation. *Hum Reprod Oxf Engl*. 2011;26(2):480–90.
- Capalbo A, Wright G, Elliott T, Ubaldi FM, Rienzi L, Nagy ZP. FISH reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. *Hum Reprod Oxf Engl*. 2013;28(8):2298–307.

34. Capalbo A, Ubaldi FM, Rienzi L, Scott R, Treff N. Detecting mosaicism in trophectoderm biopsies: Current challenges and future possibilities. *Hum Reprod Oxf Engl.* 2017;32(3):492–8.
35. Magli MC, Pomante A, Cafueri G, Valerio M, Crippa A, Ferraretti AP, et al. Preimplantation genetic testing: Polar bodies, blastomeres, trophectoderm cells, or blastocoelic fluid? *Fertil Steril.* 2016;105(3):676–683.e5.
36. Poli M, Ori A, Child T, Jaroudi S, Spath K, Beck M, et al. Characterization and quantification of proteins secreted by single human embryos prior to implantation. *EMBO Mol Med.* 2015;7(11):1465–79.
37. Gianaroli L, Magli MC, Pomante A, Crivello AM, Cafueri G, Valerio M, et al. Blastocentesis: A source of DNA for preimplantation genetic testing. Results from a pilot study. *Fertil Steril.* 2014;102(6):1692–1699.e6.
38. Palini S, Galluzzi L, De Stefani S, Bianchi M, Wells D, Magnani M, et al. Genomic DNA in human blastocoele fluid. *Reprod Biomed Online.* 2013;26(6):603–10.
39. Zhang Y, Li N, Wang L, Sun H, Ma M, Wang H, et al. Molecular analysis of DNA in blastocoele fluid using next-generation sequencing. *J Assist Reprod Genet.* 2016;33(5):637–45.
40. Capalbo A, Romanelli V, Patassini C, Poli M, Girardi L, Giancani A, et al. Diagnostic efficacy of blastocoel fluid and spent media as sources of DNA for preimplantation genetic testing in standard clinical conditions. *Fertil Steril.* 2018;110(5):870–879.e5.
41. Galluzzi L, Palini S, Stefani SD, Andreoni F, Primiterra M, Diotallevi A, et al. Extracellular embryo genomic DNA and its potential for genotyping applications. *Future Sci OA.* 2015;1(4):FSO62.
42. Zimmerman RS, Jalas C, Tao X, Fedick AM, Kim JG, Pepe RJ, et al. Development and validation of concurrent preimplantation genetic diagnosis for single gene disorders and comprehensive chromosomal aneuploidy screening without whole genome amplification. *Fertil Steril.* 2016;105(2):286–94.
43. Tobler KJ, Zhao Y, Ross R, Benner AT, Xu X, Du L, et al. Blastocoel fluid from differentiated blastocysts harbors embryonic genomic material capable of a whole-genome deoxyribonucleic acid amplification and comprehensive chromosome microarray analysis. *Fertil Steril.* 2015;104(2):418–25.
44. Magli MC, Albanese C, Crippa A, Tabanelli C, Ferraretti AP, Gianaroli L. Deoxyribonucleic acid detection in blastocoelic fluid: A new predictor of embryo ploidy and viable pregnancy. *Fertil Steril.* 2019;111(1):77–85.
45. Tsuiko O, Zhigalina DI, Jatsenko T, Skryabin NA, Kanbekova OR, Artyukhova VG, et al. Karyotype of the blastocoel fluid demonstrates low concordance with both trophectoderm and inner cell mass. *Fertil Steril.* 2018;109(6):1127–1134.e1.
46. Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, et al. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: Genetic contamination identified. *Fertil Steril.* 2017;107(1):220–228.e5.
47. Rubio C, Navarro-Sánchez L, García-Pascual CM, Ocalí O, Cimadomo D, Venier W, et al. Multicenter prospective study of concordance between embryonic cell-free DNA and trophectoderm biopsies from 1301 human blastocysts. *Am J Obstet Gynecol.* 2020;223(5):751.e1–751.e13.
48. Leaver M, Wells D. Non-invasive preimplantation genetic testing (niPGT): The next revolution in reproductive genetics? *Hum Reprod Update.* 2020;26(1):16–42.
49. Shamoni MI, Jin H, Haimowitz Z, Liu L. Proof of concept: Preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. *Fertil Steril.* 2016;106(6):1312–8.
50. Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Hum Reprod Oxf Engl.* 2018;33(4):745–56.
51. Rubio C, Rienzi L, Navarro-Sánchez L, Cimadomo D, García-Pascual CM, Albricci L, et al. Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: Concordance rate and clinical implications. *Fertil Steril.* 2019;112(3):510–9.
52. Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci U S A.* 2016;113(42):11907–12.
53. Fang R, Yang W, Zhao X, Xiong F, Guo C, Xiao J, et al. Chromosome screening using culture medium of embryos fertilised in vitro: A pilot clinical study. *J Transl Med.* 2019;17(1):73.
54. Ho JR, Arrach N, Rhodes-Long K, Ahmady A, Ingles S, Chung K, et al. Pushing the limits of detection: Investigation of cell-free DNA for aneuploidy screening in embryos. *Fertil Steril.* 2018;110(3):467–475.e2.
55. Feichtinger M, Vaccari E, Carli L, Wallner E, Mädel U, Figl K, et al. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: A proof-of-concept pilot study. *Reprod Biomed Online.* 2017;34(6):583–9.
56. Capalbo A, Ubaldi FM, Cimadomo D, Maggiulli R, Patassini C, Dusi L, et al. Consistent and reproducible outcomes of blastocyst biopsy and aneuploidy screening across different biopsy practitioners: A multicentre study involving 2586 embryo biopsies. *Hum Reprod Oxf Engl.* 2016;31(1):199–208.

14

ANALYSIS OF FERTILIZATION

Thomas Ebner

Introduction

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles have shown that women have only a finite number of gametes out of a pool of collected oocytes that are viable for generating a term pregnancy. This demonstrates the need for simple methods of pre-implantation embryo assessment in the prediction of pregnancy rates. In this respect, intensive research has been done at the zygote stage on day 1 of pre-implantation development.

Independently of the mode of fertilization, a sperm-borne enzyme called phospholipase C- ζ enters the oocyte and activates it via the inositol-3-phosphate pathway. In detail, this molecule binds to the corresponding receptor at the endoplasmic reticulum where it causes Ca^{2+} release in the form of oscillations. This Ca^{2+} response drives the extrusion of the second polar body and the formation of both pronuclei.

While conventional IVF more or less mimics natural fertilization, ICSI is a rather invasive procedure circumventing some of the major steps in the process of oocyte activation and fertilization. Consequently, the ICSI schedule differs slightly from the IVF one [1]. This delay is attributed to the time needed for the sperm to pass through the oocyte outer complex, particularly the cumulus and corona cells, along with the zona pellucida. Fusion of the spermatozoon with the oolemma and incorporation into the oocyte plasma, on the other hand, seem to occur very rapidly [2] via an orchestrated interaction between Izumo-1 (sperm) and Juno (egg) receptor proteins [3]. In ICSI, fertilization usually has to be assessed approximately two hours earlier (e.g. 16–18 hours post-insemination) than in IVF (18–20 hours post-insemination) in order to find identical developmental stages [4].

Timing of fertilization events

Either active propulsion (conventional IVF) or direct deposition (ICSI) ensures presence of a spermatozoon in the cytoplasm. There is evidence from time-lapse imaging studies indicating that regular fertilization follows a definite course of events, though the timing of these events may vary between eggs [5, 6]. Time-lapse technique further allows for the annotation of time specific morphological changes during oocyte activation, fertilization, and further embryo development which is referred to as morphokinetics.

With respect to this, a time-lapse user group proposed guidelines on the nomenclature of human embryo development, including the dynamic fertilization process [7]. Per the definition, the time at which insemination occurs (IVF or ICSI) is called t0. Consequently, tPB2 marks the time at which the second polar body is extruded and tPN marks the time at which the fertilization status is confirmed. For proper analysis of the time period in which the two pronuclei are visible (VP), their appearance (tPNa) and fading (tPNf) should be documented. It is important

to note the time of time-lapse pronuclear assessment (tZ) since the pronuclear pattern is a dynamic event and its morphology can change between tPNa and tPNf [8].

Approximately 90% of the oocytes showed circular waves of granulation within the cytoplasm [5] after ICSI. During this granulation phase, the head of the spermatozoon decondensed. Subsequently, the second polar body was extruded. A characteristic fertilization cone, probably reflecting an interaction between the male chromatin and the oocyte's cortex, was not always observed [6]. The next steps would involve the central formation of the male pronucleus and the peripheral formation of the female counterpart. The latter was then drawn towards the male pronucleus until the two abutted. Data from the literature suggest [9] that during this process the male pronucleus rotates onto the female one, in which the chromatin condenses on the side facing the centre of the egg, in order to also align its chromatin towards the spindle forming between both pronuclei. Both pronuclei then increase in size, and their nucleoli move around and arrange themselves near the common junction [5].

Within both nuclei, nucleoli form at sites on the DNA known as the "nucleolar organizing regions" located on the chromosomes where the ribosomal genes are situated [10]. This means that the nucleoli are the active sites of rRNA synthesis. During the course of development, nucleoli tend to fuse due to an increase in protein synthesis [5, 11]. It should be emphasized once again that IVF zygotes reach the final stage of nucleolar organization at a later time than ICSI zygotes.

The size and distribution patterns of the nucleoli may serve as prognostic parameters of the events of fertilization, the completion of meiosis, and the cell cycle, leading to the first mitotic division, the normality of the chromatin complement in the two nuclei, and the formation with chromosome attachment of the mitotic spindle [10].

In particular, asynchrony in formation and polarization of nucleoli (Figure 14.1a) may severely impair further development of the pre-implantation embryo [12–16]. Consequently, good-quality embryos can arise from oocytes that had more uniform timing from injection to pronuclear abutment [5].

Pronuclear grading

According to the aforementioned agreement [5], pronuclear pattern assessment using time-lapse imaging should be done immediately before tPBf. However, embryologists are faced with several pronuclear patterns at the time of fertilization assessment. Based on original data from Wright et al. [17], Scott and Smith [13] were the first to attribute zygote morphology with a certain prognostic value for subsequent implantation. In particular, the alignment of nucleoli at the junction of the two pronuclei was found to be a selection criterion for embryo transfer. Since this zygote score did not exclusively rely on the pronuclear pattern but also comprised multiple other parameters, including the appearance

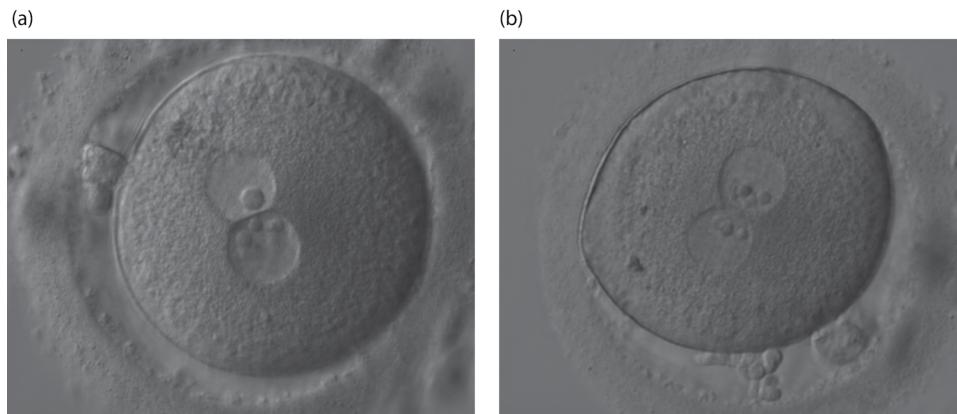


FIGURE 14.1 (a) Bad-prognosis zygote with an asymmetric pronuclear pattern corresponding to pattern 4 [14] or Z3 [16]. (b) Zygote showing optimal pronuclear pattern 0B [14] or Z1 [16] and a clear halo.

of cytoplasm and timing of nuclear membrane breakdown, the actual impact of pronuclear morphology on further outcomes remained unclear.

Thus, Tesarik and Greco [14] were the pioneers in predicting pre-implantation development by focusing exclusively on the number and distribution of nucleoli (nucleolar precursor bodies [NPBs]) in each pronucleus. They considered inter-pronuclear synchrony, evaluated 12–20 hours post-IVF/ICSI, as being more important than the actual NPB polarity at the site of pronuclear apposition since they presumed that polarization of nucleoli is not evident from the beginning of pronuclei formation, but rather appears progressively with time [11]. According to Tesarik and Greco [14], the optimal synchronized pattern 0 yields 37.3% good-quality embryos compared to all other patterns (27.8%). In addition, the frequency of developmental arrest of pattern 0 zygotes was only 8.5% as compared with 25.6% in the other patterns.

Since all these previous reports were of retrospective character, particular importance must be assigned to a prospective multi-centre study of Montag and Van der Ven [4]. These authors highlighted that cycles with transfer of at least one embryo derived from pattern 0B (Figure 14.1b), but not pattern 0A, resulted in significantly higher rates of pregnancy (37.9%) and implantation (20.5%) than nonpattern 0B cycles (26.4% and 15.7%). Similar results have been published by others [15] who found significantly increased pregnancy rates (44.8% vs. 30.2%) if embryos derived from zygotes with pattern 0 were transferred. Obviously, NPB polarization at the area of pronuclear contact outdoes pronuclear symmetry.

Scott et al. [16, 18] further refined their score by also creating a single observation zygote score. This so-called Z-score was comparable with the score introduced by Tesarik and Greco [14], since patterns Z1 and Z2 resemble patterns 0B and 0A. Several other authors successfully used the zygote scores of Scott et al. [13, 15] and Tesarik and Greco [14] for prognostic purposes [4, 15, 19–22]. Though the grading systems differ slightly in some of these papers, the conclusion is a common one. Zygotes showing pronuclei with approximately the same number and alignment of NPBs in the furrow between the nuclei had the best prognosis in terms of subsequent implantation.

It is noteworthy that Salumets et al. [23] failed to show any correlation between zygote score and pregnancy rate. This is of particular interest because this group only analysed single-embryo transfers and, consequently, the actual implantation potential

could be accurately estimated. Though two different scores were applied [13, 14], no correlation to treatment outcome could be demonstrated. This discrepancy in literature results may be explained by the use of different culture media and stimulation protocols and differences in timing of fertilization assessments (e.g. the inclusion of early cleavage in the Scott and Smith [13] scoring system).

An increased incidence of subsequent blastocyst formation in zygotes with optimal patterns of the pronuclei [16, 21, 24] seems to be consistent with the reported increase in terms of pregnancy rate. Theoretically, a lower blastocyst formation rate in abnormal zygotes could be related to their chromosomal status since there is information from the literature that several pronuclear patterns seem to be associated with aneuploidy [25–28].

In detail, Kahraman and colleagues [27] found a 52.2% rate of chromosomal abnormality in biopsied embryos derived from suspicious zygotes (showing an asymmetric distribution of NPBs), which was significantly lower than the observed 37.6% in the normal control zygotes. Others [28] also confirmed that the position of pronuclei within the cytoplasm, the size and distribution of nucleoli, and the orientation of polar bodies with respect to pronuclei were highly predictive of the presence of chromosomal abnormalities in the corresponding embryos. In this study [28], zygotes with abutted pronuclei, large-sized nucleoli, and polar bodies with small angles subtended by pronuclei and polar bodies were the configurations associated with the highest rates of euploidy. Using the Z-score, it could be shown [26] that Z1 patterns had a significantly higher rate of euploidy (71%) as compared to Z3 (35%) and Z4 (36%) patterns. The same also holds true for the score of Tesarik and Greco [14], since pattern 0 was associated with a minimal rate of aneuploidy (26%), whereas patterns with poor prognosis showed higher rates of up to 83% [25].

It is important to note that not all studies published to date suggest complete reliance on zygote morphology [23, 29]. One problem is that overall up to 14 different zygote scoring systems have been published so far. On the basis of those papers that made their way into a recent meta-analysis [30], it can be concluded that there is a lack of conclusive data on the clinical efficacy of zygote scoring.

Further evidence on the limited potential of pronuclear scoring comes from time-lapse imaging since none of the tested scoring systems [18, 31] were shown to predict the live birth outcome [8]. On the other hand, tPNf occurred significantly later in embryos

resulting in live birth and was never observed earlier than at 20 hours and 45 minutes [8].

A definite difference between IVF and ICSI cycles with regard to the frequency of good patterns (pattern 0 according to Tesarik and Greco [14]) was reported [4]. In particular, superior pronuclear patterns were observed in ICSI cycles. This phenomenon may be due to the aforementioned accelerated course of development in ICSI [1, 32]. Zygotes showing this most advanced stage of nuclear polarization seem to reach that stage earlier after ICSI than after conventional IVF [4].

However, the study did not evaluate the position of the pronuclei relative to the presumed polar axis. This arrangement has been reported to relate to embryo quality [33, 34]. Edwards and Beard (35) suggested that the oocyte may establish this polarity by either ooplasmic or pronuclear rotation towards the second polar body. Such a resetting of a new axis after fertilization is governed by cytoplasmic contraction waves organized by the sperm centrosome [35]. Embryos unable to achieve optimal pronuclear orientation, possibly due to shorter cytoplasmic waves [5], may exhibit poor morphology (e.g. uneven cleavage or fragmentation) [33].

Abnormal pronuclear formation and patterns

Single-pronucleate (1PNs) zygotes can be obtained following IVF and ICSI at frequencies ranging from 2% to 5% (36). They were reported to show a trend towards higher frequency in ICSI [36].

Karyotyping indicated that following IVF more than half of 1PN embryos are in fact diploid, but these studies [37, 38] did not differentiate between diploidy produced by fusion of both pronuclei or fertilization by parthenogenetic activation. However, in further studies it could be demonstrated that when embryos were diploid, approximately half of them were fertilized [37, 38]. Two mechanisms could be responsible for this observation: asynchronous appearance/fading [39] or fusion of both pronuclei [40]. If there is no other choice, such IVF embryos could be considered for transfer, particularly if the single pronucleus is larger than regular size. With respect to this, a recent time-lapse study [41] identified a cut-off value for the single pronuclear area ($713 \mu\text{m}^2$) and diameter ($31 \mu\text{m}^2$), which allowed to distinguish between those 1PN-zygotes that made it to blastocyst stage and those which did not (AUC 0.662 and 0.661, respectively). For the prediction of blastocyst quality AUC was even higher, e.g. 0.848 and 0.827 [41]. Of note, IVF 1PN zygotes performed better as compared to the ICSI counterpart [41].

Recent data [42] on PGT-A cycles challenge the dogma that 1PN zygotes derived from ICSI should rather not be transferred [43, 44] since close to 70% of such abnormally fertilized oocytes turned out to be diploid and some of them did result in live births.

The presence of 3PN zygotes after IVF is the most common fertilization anomaly in humans. This is mostly caused by dispermy (3PN, two polar bodies), and the majority of the corresponding embryos will cleave but stop development at later stages [36]. In ICSI, some 4% [36] of zygotes show digynic triploidy, meaning that a single sperm is present in the egg but the second polar body was not extruded (non-disjunction). In this case, the chromosomes of the three pronuclei are organized in a single bipolar spindle at syngamy, indicating that only one centrosome deriving from one sperm is active. Time-lapse imaging has shown that close to



FIGURE 14.2 Zygote showing two pronuclei with an additional smaller nucleus (2 o'clock position) possibly containing chromosomal material.

75% of tripromuclear zygotes directly cleave into three cells [45], a phenomenon which is known as trichotomous mitosis (6).

Within 3PN zygotes, a special case is the presence of 2PNs with a third additional small nucleus which Capalbo et al. [42] referred to as 2.1PN zygotes (Figure 14.2). Since the same authors have shown that the presence of a smaller nucleus can be associated with chromosomal loss or gain, the decision to select such embryos for transfer has to be carefully weighted. Due to the sometimes-small size of these additional nuclei, there is of course a high risk of missing them during routine fertilization checks, especially when using objectives of lower magnification. Again time-lapse imaging would facilitate the scoring process and would increase the chance not to miss 2.1PN zygotes.

Peripheral positioning of pronuclei

Regardless of the pronuclear pattern that the oocyte reflects, it is generally accepted that both pronuclei should be located in the centre of the female gamete. Cytoplasmic inclusions, such as dense granularity, large refractile bodies, and/or vacuoles, may displace both pronuclei. However, this scenario can also happen in zygotes with normal homogeneous ooplasm. Any deviation from the presumed optimal central arrangement (e.g. peripheral apposition of both pronuclei) (Figure 14.3) is most likely associated with reduced developmental capacity [33]. Considering the fact that the first cleavage plane runs through the contact zone of both pronuclei, it is a frequent phenomenon that the corresponding embryo will show uneven cleavage. This scenario is more frequent in conventional IVF than in ICSI (3.3% vs. 11.8%), probably due to varying sites of sperm entrance in IVF [34] (e.g. near-spindle penetration of the zona, which in turn could force eccentric formation of pronuclei [9]).

Non-juxtaposition of pronuclei

Another problem occasionally arising during fertilization is a failure in alignment of both pronuclei (Figure 14.4), which is caused by an intrinsic defect of the cytoskeleton, or the parental centrosome may cause a complete failure in alignment [13]. While it is quite uncommon in assisted reproduction technologies (approximately 1%–2%), it is rather detrimental since the

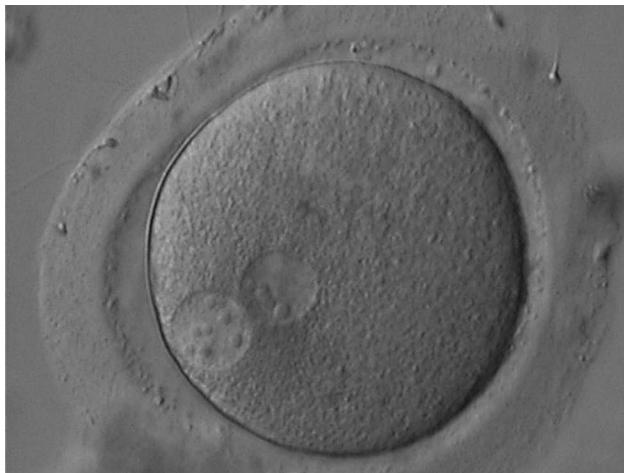


FIGURE 14.3 *In vitro* fertilization zygote showing peripheral apposition of both pronuclei.



FIGURE 14.5 Zygote with uneven pronuclear size.



FIGURE 14.4 Zygote with failure in alignment of both pronuclei.

vast majority of zygotes with unaligned pronuclei fail to cleave or show developmental arrest at early stages [14] if not resulting in chromosomal aberrations at all [28]. Morphokinetic deviations from the considered normal development includes a higher rate of trichotomous mitosis and an increased interval between tPN₁ and t2 [39].

Uneven size of pronuclei

Though the female pronucleus usually is smaller than its male counterpart [5], more extensive differences in size ($>4 \mu\text{m}$) may be observed *in vitro* (Figure 14.5). This divergence most likely is the result of problems arising during male pronucleus formation, since *in vitro*-matured oocytes from ICSI with labelled spermatozoa showed the proximity of the fluorescent sperm mid-piece remnant to the smaller pronucleus [46]. Uneven pronuclear size severely affects the viability of the corresponding embryos since more than 87% were found to be aneuploid, mostly mosaics [47, 48]. This fact probably led them to arrest at a significantly higher

rate than zygotes with pronuclear diameters showing no excessive differences. In addition, a higher incidence of day-2 multinucleation was observed [47].

Undocumented zygotes

Interestingly, 1% of all zygotes do not show pronuclei at all [36]. Manor et al. [48] demonstrated that 57% of such undocumented zygotes are normal diploid. If two polar bodies were present on day 1, corresponding embryos may be considered for transfer in case insufficient bipronucleated embryos are available. The most probable reason for this failure in detection is an abnormal developmental speed and/or inaccurate timing of fertilization control. It has also been reported that pronuclei may be hidden to extensive cytoplasmic granularity [36].

Cytoplasmic halo

Immediately prior to pronuclear growth, a microtubule-mediated withdrawal of mitochondria and other cytoplasmic components contracts from the cortex towards the centre of the oocyte, leaving a clear halo around the cortex [5]. Since the presence of a halo effect (Figure 14.1b) within the ooplasm may be recognized in 65%–85% of all zygotes [21, 23, 49], it is less applicable for scoring purposes than the pronuclear pattern. Nevertheless, this particular morphism was found to be correlated with better embryo quality [18, 23], increased blastocyst formation on day 5 [50], and a higher pregnancy rate [49].

The physiological role of mitochondrial redistribution in zygotes is still unknown, but it has been speculated that clustering of mitochondria to perinuclear regions may be involved in cell cycle regulation [51–53] (e.g. by means of calcium mobilization and ATP liberation [54–56]). In addition, location of mitochondria next to the pronuclei would allow immature mitochondria, as seen in zygotes [57], to complete maturation, presuming that some input from the nucleus is needed [53].

There is a certain disagreement between most of the studies dealing with cytoplasmic appearance at zygote stage. Some did not distinguish between several types of haloes, thus pooling symmetrical and polar haloes [13, 23], whereas others presuppose that symmetrical [49] or extreme haloes [50] are abnormal.

In view of this lack of uniformity, our working group [21] set up a prospective trial to investigate the actual influence of certain subtypes of haloes on the pre-implantation development of IVF and ICSI embryos. In this paper, haloes were measured accurately in order to see if a light or extreme halo effect would have any impact on subsequent developmental stages. Based on our findings, it was concluded that any halo effect, irrespective of its grade and dimension, is of positive predictive power in terms of blastocyst quality and, consequently, clinical pregnancy rate [21]. Neither the method used for insemination (IVF or ICSI) nor the presence of areas of dense cytoplasmic granulation or larger vacuoles affected the zygote in terms of halo performance. Furthermore, it was demonstrated that the pronuclear pattern and halo formation are two distinct parameters [21]. In contrast to the pronuclear pattern, no association between halo formation and genetic status of the fertilized egg has been observed [58].

The only available time-lapse study on the presence of the halo effect [59] reports higher rates of irregular cleavages, particularly rapid cleavage, cell fusion, and asymmetrical division in halo-negative zygotes. Additionally, the prolonged presence of the halo was associated with lower ongoing pregnancy rates.

Conclusion

During evaluation of zygote morphology, it has to be considered that both halo and pronuclear formation follow a fixed schedule. Since direct ooplasmic placement of a viable spermatozoon is performed in ICSI, thus bypassing most steps of fertilization (including acrosome reaction and zona binding), the further course of development will be somewhat accelerated as compared to conventional IVF. It is of interest that more physiological sperm selected on the basis of its potential to bind to hyaluronic acid did not influence the pronuclear score [60].

Pronuclear morphology and halo characteristics turned out to be unstable independent factors within the dynamic process of fertilization. The degree and morphology of the halo per se have no influence on further outcome. However, the presence of such a halo had positive predictive power. Consequently, halo formation in combination with optimal pronuclear patterns (e.g. those with alignment of fused nucleoli) will characterize a subgroup of oocytes showing a developmental advantage compared to zygotes lacking these positive predictors.

This is in line with recent findings indicating that during syngamy those zygotes with an accelerated breakdown of the pronuclear membranes at 22–25 hours post-insemination or post-injection implanted significantly more frequently than those with delayed dissolution [61]. This is not to forget the reported positive correlation between the occurrence of the first mitotic division and the rates of implantation and clinical pregnancy [62–64].

Recently, promising strategies have been published combining the morphological information of zygote stage with other developmental stages [65–69]. In detail, sequential assessment of cultured human embryos allowed for accurate prognosis in terms of good-quality blastocyst development [67, 68]. Others [65] found a relatively high outcome predictability after IVF using a combined score for zygote and embryo morphology and growth rate. Finally, day-3 embryo transfer with combined evaluation at the pronuclear and cleavage stage compared favourably with day-5 blastocyst transfer [66].

This suggests that zygote stage, although being an important developmental phase, should not be used solitarily as a prognostic parameter, but rather morphological information from day 1

should be pooled with that of earlier and/or later stages in order to maximize benefit and minimize the numbers of embryos transferred. The contribution of deep learning and artificial intelligence techniques to automatic detection and particularly scoring of pronuclei is not yet assessable but first publications are available [70].

References

- Nagy ZP, Janssenswillen C, Janssens R, et al. Timing of oocyte activation, pronucleus formation, and cleavage in humans after intracytoplasmic sperm injection (ICSI) with testicular spermatozoa and after ICSI or *in-vitro* fertilization on sibling oocytes with ejaculated spermatozoa. *Hum Reprod.* 1998;13:1606–12.
- van Wissen B, Wolf JP, Bomsel-Helmreich O, et al. Timing of pronuclear development and first cleavages in human embryos after subzonal insemination: Influence of sperm phenotype. *Hum Reprod.* 1995;10:642–8.
- Bianchi E, Doe B, Goulding D et al. Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature.* 2014;508(7497): 483–7.
- Montag M, Van der Ven H. Evaluation of pronuclear morphology as the only selection criterion for further embryo culture and transfer: Results of a prospective multicentre study. *Hum Reprod.* 2001;16:2384–9.
- Payne D, Flaherty SP, Barry MF, et al. Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. *Hum Reprod.* 1997;12:532–41.
- Coticchio G, Mignini Renzini M, et al. Focused time-lapse analysis reveals novel aspects of human fertilization and suggests new parameters of embryo viability. *Hum Reprod.* 2018;33:23–31.
- Ciray HN, Campbell A, Agerholm IE, et al. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. *Hum Reprod.* 2014;29: 2650–60.
- Azzarello A, Hoest T, Mikkelsen AL. The impact of pronuclei morphology and dynamicity on live birth outcome after time-lapse culture. *Hum Reprod.* 2012;27:2649–57.
- Van Blerkom J, Davis P, Merriman J, et al. Nuclear and cytoplasmic dynamics of sperm penetration, pronuclear formation and microtubule organization during fertilization and early preimplantation development in the human. *Hum Reprod Update.* 1995;1:429–61.
- Scott L. The biological basis of non-invasive strategies for selection of human oocytes and embryos. *Hum Reprod Update.* 2003;9:137–249.
- Tesarik J, Kopecny V. Development of human male pronucleus: Ultrastructure and timing. *Gamete Res.* 1989;24:135–49.
- Van Blerkom J. Occurrence and developmental consequences of aberrant cellular organization in meiotically mature oocytes after exogenous ovarian hyperstimulation. *J Electron Microsc Tech.* 1990;16:324–46.
- Scott LA, Smith S. The successful use of pronuclear embryo transfers the day following oocyte retrieval. *Hum Reprod.* 1998;13:1003–13.
- Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. *Hum Reprod.* 1999;14:1318–23.
- Tesarik J, Junca AM, Hazout A, et al. Embryos with high implantation potential after intracytoplasmic sperm injection can be recognized by a simple, noninvasive examination of pronuclear morphology. *Hum Reprod.* 2000;15:1396–99.
- Scott LA, Alvero R, Leondires M. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. *Hum Reprod.* 2000;15:2394–403.
- Wright G, Wiker S, Elsner C, et al. Observations on the morphology of pronuclei and nucleoli in human zygotes and implications for cryopreservation. *Hum Reprod.* 1990;5:109–15.

18. Scott L. Pronuclear scoring as a predictor of embryo development. Reprod Biomed Online. 2003;6:201–14.
19. Ludwig M, Schöpper B, Al-Hasani S, Diedrich K. Clinical use of a PN stage score following intracytoplasmic sperm injection: Impact on pregnancy rates under the conditions of the German embryo protection law. Hum Reprod. 2000;15:325–9.
20. Wittemer C, Bettahar-Lebugle K, Ohl J. Zygote evaluation: An efficient tool for embryo selection. Hum Reprod. 2000;15:2591–7.
21. Ebner T, Moser M, Sommergruber M, et al. Presence, but not type or degree of extension, of a cytoplasmic halo has a significant influence on preimplantation development and implantation behaviour. Hum Reprod. 2003;18:2406–12.
22. Arroyo G, Veiga A, Santaló J, et al. Developmental prognosis for zygotes based on pronuclear pattern: Usefulness of pronuclear scoring. *J Assist Reprod Genetics*. 2007;14:173–81.
23. Salumets A, Hydén-Granskog C, Suikkari AM, et al. The predictive value of pronuclear morphology of zygotes in the assessment of human embryo quality. Hum Reprod. 2001;16:2177–81.
24. Balaban B, Urman B, Isiklar A, et al. The effect of pronuclear morphology on embryo quality parameters and blastocyst transfer outcome. Hum Reprod. 2001;16:2357–61.
25. Balaban B, Yakin K, Urman B, et al. Pronuclear morphology predicts embryo development and chromosome constitution. Reprod Biomed Online. 2004;8:695–700.
26. Chen CK, Shen GY, Horng SG, et al. The relationship of pronuclear stage morphology and chromosome status at cleavage stage. *J Assist Reprod Genetics*. 2003;20:413–20.
27. Kahraman S, Kumtepe Y, Sertiyel S, et al. Pronuclear morphology scoring and chromosomal status of embryos in severe male infertility. Hum Reprod. 2002;17:3193–200.
28. Gianaroli L, Magli MC, Ferraretti AP, et al. Pronuclear morphology and chromosomal abnormalities as scoring criteria for embryo selection. Fertil Steril. 2003;80:341–9.
29. Arroyo G, Santaló J, Parriego M, et al. Pronuclear morphology, embryo development and chromosome constitution. Reprod Biomed Online. 2010;20:649–55.
30. Nicoli A, Palomba S, Capodanno F, et al. Pronuclear morphology evaluation for fresh *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles: A systematic review. *J Ovarian Res*. 2013;6:12–64.
31. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. Hum Reprod. 2011;26:1270–83.
32. Sakkas D, Shoukir Y, Chardonnens D, et al. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum Reprod. 1998;13:182–7.
33. Garello C, Baker H, Rai J, et al. Pronuclear orientation, polar body placement, and embryo quality after intracytoplasmic sperm injection and *in-vitro* fertilization: Further evidence for polarity in human oocytes? Hum Reprod. 1999;14:2588–94.
34. Kattera S, Chen C. Developmental potential of human pronuclear zygotes in relation to their pronuclear orientation. Hum Reprod. 2004;19:294–9.
35. Edwards RG, Beard HK. Oocyte polarity and cell determination in early mammalian embryos. Mol Hum Reprod. 1997;3:863–905.
36. Munné S, Cohen J. Chromosome abnormalities in human embryos. Hum Reprod Update. 1997;6:842–55.
37. Plachot M. Chromosome analysis of oocytes and embryos. In: Preimplantation Genetics. Verlinsky Y, Kuliev A (eds). New York, NY: Plenum Press, pp. 103–12, 1991.
38. Staessen C, Janssenwillen C, Devroey P, et al. Cytogenetic and morphological observations of single pronucleated human oocytes after *in-vitro* fertilization. Hum Reprod. 1993;8:221–3.
39. Ezoe K, Coticchio G, Takenouchi H, et al. Spatiotemporal perturbations of pronuclear breakdown preceding syngamy affect early human embryo development: A retrospective observational study. *J Assist Reprod Genet*. 2022;39(1):75–84. doi: [10.1007/s10815-021-02335-6](https://doi.org/10.1007/s10815-021-02335-6).
40. Sultan KM, Munné S, Palermo GD, et al. Chromosomal status of uni-pronuclear human zygotes following *in-vitro* fertilization and intracytoplasmic sperm injection. Hum Reprod. 1995;10:132–6.
41. Araki E, Itoi F, Honnma H, et al. Correlation between the pronucleus size and the potential for human single pronucleus zygotes to develop into blastocysts: 1PN zygotes with large pronuclei can expect an embryo development to the blastocyst stage that is similar to the development of 2PN zygotes. *J Assist Reprod Genet*. 2018;35:817–23.
42. Capalbo A, Treff N, Cimadomo D, et al. Abnormally fertilized oocytes can result in healthy live births: Improved genetic technologies for preimplantation genetic testing can be used to rescue viable embryos in *in vitro* fertilization cycles. Fertil Steril. 2017;108:1007–1015.e3.
43. Staessen C, Van Steirteghem AC. The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional *in-vitro* fertilization. Hum Reprod. 1997;12:321–7.
44. Levron J, Munné S, Willadsen S, et al. Male and female genomes associated in a single pronucleus in human zygotes. Biol Reprod. 1995;52:653–7.
45. Joergensen MW, Agerholm I, Hindkjaer J, et al. Altered cleavage patterns in human tripornuclear embryos and their association to fertilization method: A time-lapse study. *J Assist Reprod Genet*. 2014;31:435–442.
46. Goud P, Goud A, Van Oostveldt P, et al. Fertilization abnormalities and pronucleus size asynchrony after intracytoplasmic sperm injection are related to oocyte post maturity. Fertil Steril. 1999;72:245–52.
47. Sadawy S, Tomkin G, Munné S, et al. Impaired development of zygotes with uneven pronuclear size. Zygote. 1998;6:137–41.
48. Manor D, Drugan A, Stein D, et al. Unequal pronuclear size—A powerful predictor of embryonic chromosome anomalies. *J Assist Reprod Genetics*. 1999;16:385–9.
49. Stalf T, Herrero J, Mehnert C, et al. Influence of polarization effects and pronuclei on embryo quality and implantation in an IVF program. *J Assist Reprod Genetics*. 2002;19:355–62.
50. Zollner U, Zollner KP, Hartl G, et al. The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts: The German experience. Hum Reprod. 2002;17:1327–33.
51. Barnett DK, Kimura J, Bavister BD. Translocation of active mitochondria during hamster preimplantation embryo development studied by confocal laser scanning microscopy. Dev Dyn. 1996;205:64–72.
52. Wu GJ, Simerly C, Zoran SS, et al. Microtubule and chromatin dynamics during fertilization and early development in rhesus monkeys, and regulation by intracellular calcium ions. Biol Reprod. 1996;55:260–70.
53. Bavister BD, Squirrell JM. Mitochondrial distribution and function in oocytes and early embryos. Hum Reprod. 2000;15(Suppl 2):189–98.
54. Sousa M, Barros A, Silva J, et al. Developmental changes in calcium content of ultrastructurally distinct subcellular compartments of pre-implantation human embryos. Mol Hum Reprod. 1997;3:83–90.
55. Diaz G, Setzu M, Zucca A, et al. Subcellular heterogeneity of mitochondrial membrane potential: Relationship with organelle distribution and intercellular contacts in normal, hypoxic and apoptotic cells. *J Cell Sci*. 1999;112:1077–84.
56. Van Blerkom J, Davis P, Alexander S. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: Relationship to microtubular organization, ATP content and competence. Hum Reprod. 2000;15:2621–33.
57. Motta PM, Nottola SA, Makabe S, et al. Mitochondrial morphology in human fetal and adult gene cells. Hum Reprod. 2000;15(Suppl):129–47.

58. Coskun S, Hellani A, Jaroudi K, et al. Nuclear precursor body distribution in pronuclei is correlated to chromosomal embryos. *Reprod Biomed Online.* 2003;7:86–90.
59. Ezoe K, Hickman C, Miki T et al. Cytoplasmic halo characteristics during fertilization and their implications for human preimplantation embryo development and pregnancy outcome. *Reprod Biomed Online.* 2020;41:1 91–202.
60. Van den Bergh M, Fahy-Deshe M, Hohl MK. Pronuclear zygote score following intracytoplasmic injection of hyaluronan-bound spermatozoa: A prospective randomized study. *Reprod Biomed Online.* 2009;19:796–801.
61. Fancsovits P, Toth L, Takacz ZF, et al. Early pronuclear breakdown is a good indicator of embryo quality and viability. *Fertil Steril.* 2005;84:881–7.
62. Shoukir Y, Campana A, Farley T, et al. Early cleavage of *in-vitro* fertilized human embryos to the 2-cell stage: A novel indicator of embryo quality and viability. *Hum Reprod.* 1997;12:1531–6.
63. Sakkas D, Percival G, D'Arcy Y, et al. Assessment of early cleaving in *in vitro* fertilized human embryos at the 2-cell stage before transfer improves embryo selection. *Fertil Steril.* 2001;76:1150–6.
64. Lundin K, Bergh C, Hardarson T. Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Hum Reprod.* 2001;16:2652–7.
65. De Placido G, Wilding M, Strina I, et al. High outcome predictability after IVF using a combined score for zygote and embryo morphology and growth rate. *Hum Reprod.* 2002;17:2402–9.
66. Rienzi L, Ubaldi F, Iacobelli M, et al. Day 3 embryo transfer with combined evaluation at the pronuclear and cleavage stage compares favourably with day 5 blastocyst transfer. *Hum Reprod.* 2002;17:1852–5.
67. Lan KC, Huang FJ, Lin YC, et al. The predictive value of using a combined Z-score and day 3 embryo morphology score in the assessment of embryo survival on day 5. *Hum Reprod.* 2003;18:1299–306.
68. Neuber E, Rinaudo P, Trimarchi JR, et al. Sequential assessment of individually cultured human embryos as an indicator of subsequent good quality blastocyst development. *Hum Reprod.* 2003;18:1307–12.
69. Alvarez C, Taranger R, Garcia-Garrido C, et al. Zygote score and status 1 or 2 days after cleavage and assisted reproduction outcome. *Int J Gynaecol Obstet.* 2008;101:16–20.
70. Fukunaga N, Sanami S, Kitasaka H, et al. Development of an automated two pronuclei detection system on time-lapse embryo images using deep learning techniques. *Reprod Med Biol.* 2020;19:286–94.

15

CULTURE SYSTEMS FOR THE HUMAN EMBRYO

David K. Gardner

Introduction

Embryo culture is often mistaken for a relatively simple procedure, when in reality it is a complex task, requiring a high level of training for embryologists, together with proactive quality control and quality assurance programs to ensure the optimum performance of the laboratory and equipment. Further, a sufficient number of suitable workstations and incubation chambers are required to maintain a stable environment for embryo development *in vitro*. Evidently, embryo culture is far more involved than simply using the appropriate culture media formulations. Consequently, in order to optimize embryo development *in vitro* and maintain viability to ensure the delivery of a healthy baby, it is essential to consider embryo culture as a system in its entirety. The embryo culture system consists of the media, macromolecules, gas phase, type of medium overlay, the culture vessel, the incubation chamber, ambient air quality and even the embryologists themselves. The concept of an embryo culture system exemplifies the interactions that exist not only between the embryo and its physical surroundings, but between all parameters within the laboratory (Figure 15.1) [1]. Only by taking such a holistic approach can one optimize embryo development *in vitro* and maintain success rates.

Working *in vitro*, literally “in glass,” means that stressors are present in the culture system, which are not present within the lumen of the female reproductive tract. Stressors identified in the embryology laboratory which can have a negative impact on gametes and embryos include: transient temperature shifts as gametes and embryos are manipulated, increases in medium osmolarity if the medium is not covered with an oil overlay, changes to the levels of carbon dioxide and hence changes in pH when embryos are taken in and out of an incubator, potential physical stress should pipetting be too vigorous, atmospheric oxygen (even transient exposures are detrimental and cumulative), and the accumulation of ammonium from amino acids [2]. Of practical significance, the earlier stages of development, particularly prior to compaction, are the most susceptible to such stressors, and this is represented schematically in Figure 15.2. Furthermore, stressors have the capacity to interact with each other and create negative synergies which has significant adverse consequences for embryo development and viability [2, 3].

Finally, it is important to appreciate that it is not feasible to make a good embryo from poor quality gametes (current investigations on oocyte rejuvenation through mitochondrial transfer notwithstanding). Rather, the role of the laboratory is to maintain the inherent viability of the oocyte and sperm from which the embryo is derived. Hence, the success of IVF is dependent on the quality of the ovarian stimulation determined by the physician, the preparation/development of a receptive endometrium, as well as on patient factors including the impact of their lifestyle choices (especially diet), hence emphasizing the need for a broader perspective of patient management as well as laboratory

management. Consequently, in order to ensure consistent successful outcomes, it is paramount that appropriate communication pathways exist between physicians and scientists to ensure all variables are considered and discussed, and that action plans are in place, so that changes can be rapidly implemented in response to any concerns.

The human embryo in culture

Serendipitously for the development of human IVF, the human embryo exhibits a considerable degree of plasticity, enabling it to develop under a wide variety of culture conditions. Indeed, it appears that the human preimplantation embryo is the most resilient of all mammalian species studied to date with regards to its ability to tolerate a range of culture environments. However, this should be perceived as a reflection of the ability of the human embryo to adapt to its surroundings and not our ability to culture it. Undoubtedly, having to adapt to sub-optimal collection and/or culture conditions comes at the cost of impaired viability and potentially compromised pregnancy outcomes [2, 4, 5]. Therefore, it is important to focus on the generation of healthy embryos, as it is clear that embryo development in culture, even to the blastocyst stage per se, does not necessarily equate to the development of a viable embryo [1, 6, 7]. Implantation rate (fetal heart rate) is a key parameter utilized to evaluate the performance of the IVF laboratory in this regard, as it provides relatively quick information on cycle performance. However, the definition of viability is best defined as the ability of the embryo to implant successfully and give rise to a normal healthy term baby. Hence, only live birth rates and cumulative pregnancy rate per retrieval reflect the true efficacy of an IVF laboratory.

Today, clinics are not only faced with a multitude of embryo culture media to choose from and what gas phases to employ, but also with the decision of whether to transfer at the cleavage or the blastocyst stage. Data accumulated over the past 25 years indicate an increase in pregnancy and implantation rates and reduced pregnancy loss following blastocyst culture [8, 9]. This, combined with a move to perform preimplantation genetic screening through trophectoderm biopsy and next generation sequencing, and embryo vitrification at the blastocyst stage [10], means it is important that laboratories are able to support extended culture. It is therefore the aim of this chapter to discuss the types of media and culture systems currently available to support viable blastocyst development, and to describe how such systems can be successfully implemented in a clinical setting.

Significance of single embryo transfer for the laboratory

It is evident that with the development of enhanced culture systems and better methods for embryo selection (see Chapter 16 by Sakkas and Gardner) [7] and cryopreservation (Chapters 20

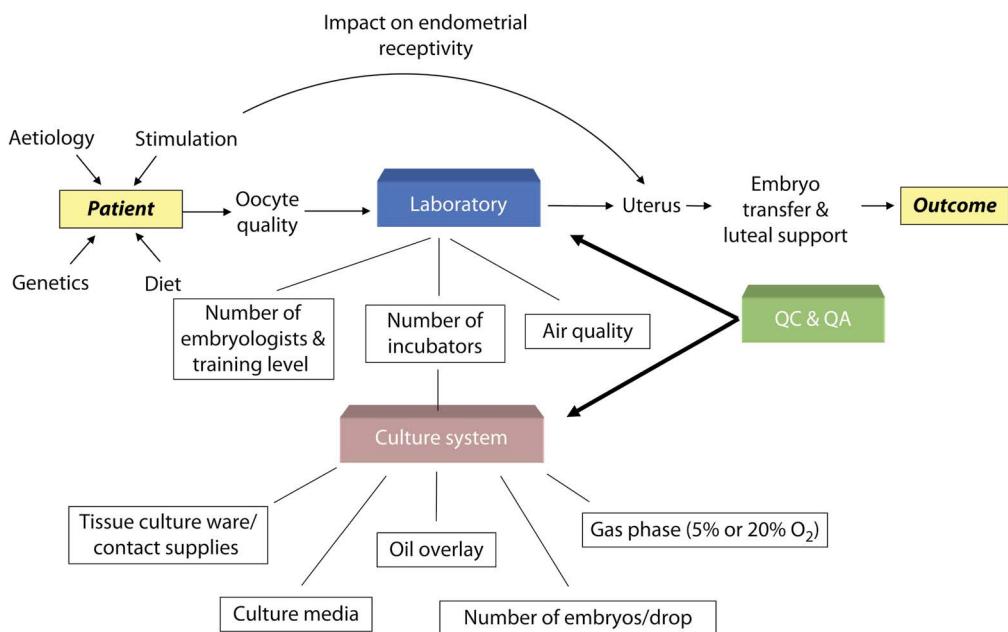


FIGURE 15.1 Holistic analysis of the human IVF laboratory and transfer outcome. This figure serves to illustrate the complex and interdependent nature of human IVF treatment. For example, the stimulation regimen used not only impacts on oocyte quality (and hence embryo physiology and viability [245]), but can also affect subsequent endometrial receptivity [186, 246–248]. Furthermore, the health and dietary status of the patient can have a profound effect on the subsequent developmental capacity of the oocyte and embryo [249, 250]. The dietary status of patients attending IVF is typically not considered as a compounding variable but growing data would indicate otherwise. In the schematic, the laboratory has been broken down into its core components, only one of which is the culture system. The culture system has in turn been broken down to its components, only one of which is the culture media. Therefore, it would appear rather simplistic to assume that by changing only one part of the culture system (i.e. culture media), that one is going to mimic the results of a given laboratory or clinic. One of the biggest impacts on the success of a laboratory and culture system is the level of quality control and quality assurance in place. For example, one should never assume that anything coming into the laboratory that has not been pre-tested with a relevant bioassay (e.g. mouse embryo assay), is safe merely because a previous lot has performed satisfactorily. Only a small percentage of the contact supplies and tissue culture ware used in IVF comes suitably tested. Therefore, it is essential to assume that everything entering the IVF laboratory without a suitable pre-test is embryo toxic until proven otherwise. In our program the 1-cell mouse embryo assay (MEA) is employed to pre-screen every lot of tissue culture ware that enters the program, i.e. plastics that are approved for tissue culture. Around 25% of all such material fails the 1-cell MEA (in a simple medium lacking protein after the first 24 hours) [213]. Therefore, if one does not perform QC to this level, one in four of all contact supplies used clinically will be embryo toxic. In reality many programs cannot allocate the resources required for this level of QC and when embryo quality is compromised in the laboratory it is the media that are held responsible, when in fact the labware are more often the culprit. (Modified from [1], with permission.)

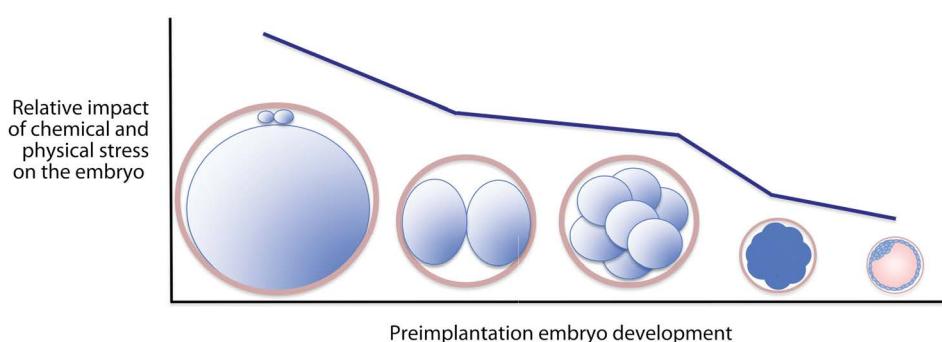


FIGURE 15.2 Relative impact of chemical and physical stress on preimplantation embryo (from oocyte to blastocyst stage), representing the stage-specific differences in the embryo's response to stress. The fertilized oocyte being more sensitive than the cleavage stage embryo, which in turn is more susceptible to stress than an embryo post-compaction. Of all stages the blastocyst is least perturbed by such factors. (From [3], with permission.)

to [22]), that single embryo transfer (SET) is the standard of care for our patients [11], thereby alleviating one of the greatest problems associated with IVE, that being multiple births [12]. One of the impacts of uptake of SET is the increased reliance the laboratory has on a successful cryopreservation program. Therefore, an important consideration in assessing the efficacy of any culture system is its ability to produce high quality embryos that can survive cryopreservation as this has a significant impact on cumulative pregnancy rates per retrieval. Of significance, the culture conditions themselves have a profound effect on cryopreservation outcome, particularly the inclusion of hyaluronan in the media, discussed later in this chapter.

Dynamics of embryo and maternal physiology

Before attempting to culture any cell type, be it embryonic or somatic, it is important to consider the physiology of the cell in order to establish its nutrient requirements. The mammalian embryo represents an intriguing scenario in that it undergoes significant changes in its physiology, molecular regulation and metabolism during the preimplantation period. The preimplantation human embryo is a highly dynamic entity which changes its needs as development proceeds. Indeed, it goes from being one of the most quiescent tissues in the body (the pronucleate oocyte), to being amongst the most metabolically active (the blastocyst) within just four days [4, 13, 14]. Interestingly, the pronucleate oocyte, like the MII oocyte from which it was derived, exhibits relatively low levels of oxygen consumption and prefers the carboxylic acid pyruvate as its primary energy source [13, 15]. Lactate can be utilized as sole substrate only from the 2-cell stage, while glucose is only consumed and utilized in relatively small amounts by the cleavage stage embryo [16]. The balance of mitochondrial and cytoplasmic metabolism is critical at these early stages of development to maintain adequate levels of ATP production [17]. However, despite the low levels of biosynthetic activity at these early stages of development there is an increasing awareness of a significant amount of remodelling of the nucleus. For example, there are major changes in methylation and acetylation levels, with many of the processes involved still to be elucidated [18–20]. Nevertheless, what is critical is that many key developmental events, such as activation of the egg and regulation of methylation and acetylation, are regulated by proteins whose activity is dependent on metabolic activity and appropriate regulation [21–24]. Therefore, maintenance of metabolic homeostasis at these early stages is paramount for the maintenance of viability (Figure 15.2).

As development proceeds and energy demands increase with cell multiplication, transcription following activation of the embryonic genome, subsequent increases in protein synthesis and the formation of the blastocoel, there is a concomitant increase in energy requirement and in glucose utilization. By the blastocyst stage, the embryo exhibits high oxygen utilization and an ability to readily utilize glucose, along with other energy sources. However, in spite of high levels of oxygen consumption, the blastocyst produces significant amounts of lactate, a phenomenon known as aerobic glycolysis, also referred to as the Warburg effect after Otto Warburg who first characterized this idiosyncratic metabolism in cancers [25]. As an aside, Warburg proposed that the mitochondria in cancer cells were dysfunctional, hence the significant production of lactate. However, it transpires that

TABLE 15.1 Differences in Embryo Physiology Pre- and Post-Compaction

Pre-Compaction	Post-Compaction
Low biosynthetic activity	High biosynthetic activity
Low QO ₂	High QO ₂
Pyruvate preferred nutrient	Glucose preferred nutrient
Requirement for specific amino acids including alanine, aspartate, glutamate, glycine, proline, serine and taurine	Requirement for a more comprehensive group of amino acids
Maternal genome	Embryonic genome
Individual cells	Transporting epithelium
One cell type	Two distinct cell types: ICM & Trophectoderm

the mitochondria of cancer cells can be very active, and the lactic acid produced, which creates an acidic microenvironment, is actually a means to facilitate tissue invasion, or in the case of the blastocyst to promote successful implantation [26, 27]. The mechanisms underpinning the metabolic shift during preimplantation development have been reviewed and remain an ongoing area of research [13, 14].

Table 15.1 highlights some of the differences between the pre- and post-compacted embryo. In many ways the physiology of the cells of the embryo prior to compaction, hence before the formation of a transporting epithelium, can be likened to unicellular organisms [28]. This in part explains why those amino acids present at high levels in the oviduct and classified as “non-essential” for tissue culture purposes, are beneficial to the cleavage stage embryo, as they ensure several key cell functions, as described later.

Within the human female reproductive tract, the nutrients available mirror the changing nutrient preferences of the embryo. At the time when the human embryo resides in the oviduct, the fluid within is characterized by relatively high concentrations of pyruvate (0.32 mM) and lactate (10.5 mM), and a relatively low concentration of glucose (0.5 mM) [29]. In contrast, uterine fluid is characterized by relatively low levels of pyruvate (0.1 mM) and lactate (5.87 mM), and a higher concentration of glucose (3.15 mM), consistent with the changes in embryo energy production. These nutrient gradients do not only provide appropriate stage specific energy substrates, but also provide stage specific signals important not just for metabolism but also for the control of molecular signalling [14].

Susceptibility of the preimplantation embryo to stress

Although the early embryo is highly adaptive to its environment, it does so at the cost of normal cellular processes and checkpoints that may be essential for viability. Therefore, as a result many embryos can appear to be morphologically normal while at a cellular level are actually highly perturbed and unlikely to be viable [5, 28]. It is clear from animal models, where invasive assessments allow additional insight, that disruptions to molecular pathways including stress response pathways frequently occur in the absence of any changes to embryo morphology. Furthermore, frequently these perturbations are permissive of implantation but affect subsequent fetal growth [30–32]. Consequently, a key focus

of the embryology laboratory should be to ensure its gamete collection and culture system are able to maintain normal cellular physiology in order to ensure the health of the embryo.

Cleavage stages versus post-compaction embryo and stress

As a result of its more “primitive” physiology, the pre-compaction stage embryo is highly susceptible to stress compared to the post-compaction stage embryo. A stress applied *in vitro* at the 2PN to the 8-cell stage can have harmful effects on normal cellular physiology and viability of the subsequent blastocyst and fetus (Figure 15.2) [28, 33–35]. At these early stages of development prior to activation of the embryonic genome, the embryo possesses only limited capacity at a molecular level to respond to a stress. In somatic cells, when a cell finds itself in a hostile environment it can activate a cascade of molecular signalling pathways to engage systems to maintain normal development. However, the pre-compaction stage embryo has a limited capacity for gene transcription [36] and, therefore, the human embryo prior to the 8-cell stage is highly vulnerable to any perturbed environment. Further, at these early stages of embryo development prior to compaction there is limited capacity to maintain normal cellular functions such as regulation of intracellular pH (pHi) [37, 38], alleviation of oxidative stress and ionic homeostasis [5, 28]. Therefore, a stress applied prior to compaction can result in major disruptions to subsequent viability. In contrast, the application of the same stress post-compaction (i.e. post the formation of a transporting epithelium) and post-embryonic genome activation typically has limited negative impact on subsequent developmental competence [28, 34, 35]. Additionally, stress can be masked at the level of morphological assessment and may only become evident downstream of the stress itself. For example, the detrimental effects of a stress applied at the early stage of development during handling and culture of the oocyte and 2PN may not be evident until the blastocyst stage. Effects can be at a sub-cellular level with the embryo having reduced metabolic capacity, high levels of apoptosis, and altered molecular profile, which ultimately result in a reduction in pregnancy rates [33–35]. Therefore, the conditions employed for gamete collection and culture of the human cleavage stage embryo directly affect the ability of the embryo to implant and form a viable pregnancy, independent of morphological assessments within the laboratory. The inability of morphology alone to distinguish viable and non-viable embryos highlights a major limitation in the field and reaffirms the need for the development of more diagnostic procedures to quantitate normal development [7, 39] (see Chapter 16 by Sakkas and Gardner).

Composition of culture media

There are several extensive treatises on the composition of embryo culture media [40–50], and it is beyond the scope of this chapter to discuss in detail the role of individual medium components. However, two key components, amino acids and macromolecules, will be considered briefly due to their significant impact on cycle outcome. Understanding their effects on embryo physiology will greatly assist clinics to make a more informed decision regarding their choice of culture media.

Amino acids

It is certainly the case that the human embryo can grow in the absence of amino acids. The real question is how well do they develop in their absence and how healthy are the resultant

TABLE 15.2 Functions of Amino Acids during Preimplantation Mammalian Embryo Development

Role	Reference
Biosynthetic precursors	[238]
Energy source	[239]
Regulators of energy metabolism	[4, 17]
Osmolytes	[154]
Buffers of pHi	[37]
Antioxidants	[240]
Chelators	[241]
Signalling	[242, 243]
Regulation of differentiation	[74, 244]

embryos? There are several reasons for the inclusion of amino acids in embryo culture media. Oviduct and uterine fluids contain significant levels of free amino acids [51–56], while both oocytes and embryos possess specific transport systems for amino acids [57] to maintain an endogenous pool [58]. Amino acids are readily taken up and metabolized by the embryo [59, 60]. Table 15.2 lists the roles amino acids can fulfil during the pre- and peri-implantation period of mammalian embryo development.

Oviduct and uterine fluids are characterized by high concentrations of the amino acids alanine, aspartate, glutamate, glycine, proline serine and taurine [51–56]. With the exception of taurine, the amino acids at high concentrations in oviduct fluid bear a striking homology to those amino acids present in Eagle’s non-essential amino acids [61]. Studies on the embryos of several mammalian species, such as mouse [62–65], hamster [66, 67], sheep [68, 69], cow [70, 71], and human [72, 73], have all demonstrated that the inclusion of amino acids in the culture medium enhances embryo development to the blastocyst stage.

More significantly, it has been demonstrated that the preimplantation mouse and cow embryo exhibits a switch in amino acid requirements as development proceeds. Up to 8-cell stage non-essential amino acids and glutamine increase cleavage rates, [71, 74, 75], i.e. those amino acids present at the highest levels in oviduct fluid stimulate the cleavage stage embryo. However, after compaction, non-essential amino acids and glutamine increase blastocoel formation and hatching, while the essential amino acids stimulate cleavage rates and increase development of the inner cell mass in the blastocyst [33, 74]. Importantly, amino acids have been reported to increase viability of cultured embryos from several species after transfer to recipients [40, 69, 74] along with increasing embryo development in culture. In the mouse, equivalent implantation rates to *in vivo* developed blastocysts have been achieved when pronucleate embryos were cultured with non-essential amino acids to the 8-cell stage followed by culture with all 20 amino acids from the 8-cell stage to the blastocyst [49, 74].

Of note, those amino acids classified as non-essential act as strong intracellular buffers of pH due to their zwitterionic nature [66], and are able to chelate toxins. As discussed, prior to compaction the blastomeres of the mammalian embryo appear to behave like unicellular organisms and therefore use exogenous amino acids to regulate their homeostasis. In contrast, post compaction and the generation of a transporting epithelium, the embryo is able to regulate its internal environment and is not as dependent on the non-essential amino acids to regulate intracellular function [37]. However, the terms non-essential and essential have

little meaning in terms of embryo development and differentiation, rather they reflect the requirements of certain somatic cells *in vitro* [61], and consequently their use is rather restrictive when it comes to the embryo's changing requirements. Indeed, a case can be made for the inclusion of specific essential amino acids such as methionine and cysteine during cleavage stage development, the former for its role in methylation pathways and the latter for its antioxidant capabilities (see section "Antioxidants" later in the chapter) [76]. Clearly, the process of culture media optimization remains ongoing.

Of great practical and clinical relevance is that even a transient exposure (~ five minutes) of mouse pronucleate oocytes to medium lacking amino acids impairs subsequent developmental potential [77]. During this five-minute period in a simple medium the pronucleate oocyte loses its entire endogenous pool of amino acids, which takes several hours of transport to replenish after returning the embryo to medium with amino acids. This has direct implications for the collection of oocytes, and more importantly the manipulation of denuded oocytes during ICSI, where the inclusion of amino acids in the holding medium will decrease or prevent intracellular stress (see more on this later in this chapter). Hence, media lacking amino acids should not be used for any oocyte or embryo handling or culture. Consistent with this recommendation, the work of Ho et al. [78] on gene expression in mouse embryos cultured in the presence of amino acids was comparable to that of embryos developed *in vivo*. In contrast, mouse embryos cultured in the absence of amino acids, i.e. in a medium based on a simple salt solution, exhibited aberrant gene expression and altered imprinting of the H19 gene [79].

Macromolecules

Most culture media for the human embryo contain serum albumin as the protein source. Historically, serum was employed worldwide, however the use of serum is no longer condoned due its extensive documented detrimental effects on embryos [69, 80–83].

Although serum albumin is a relatively pure fraction of blood, it is still contaminated with fatty acids and other small molecules. The latter has been shown to include an embryotrophic factor, citrate, which stimulates cleavage and growth in rabbit morulae and blastocysts [84]. Not only are there significant differences between sources of serum albumin [85, 86], but also between batches from the same source [85, 87, 88]. Therefore, when using serum albumin or any albumin preparation, it is essential that each batch is screened by the manufacturer for its ability to adequately support mouse embryo development and human sperm survival prior to clinical use. Furthermore, new concerns with regards to the use of human serum albumin have been raised since it has been revealed that serum albumin, added as the protein supplement, is the source of detectable levels of Di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate, as well as polybrominated diphenyl ethers in human embryo culture media [89, 90]. In addition to these compounds, serum albumin preparations also contain variable levels of contaminants that include, carbohydrates, amino acids, transition metals, and growth factors. These contaminants will be modifying the compositions of the base media in a way that is essentially variable between lots and uncontrolled [91]. Such data infer that the use of serum albumin in clinical IVF warrants renewed consideration.

To this end, recombinant human albumin is available, which eliminates the problems inherent in using blood derived products and could lead to the standardization of media formulations.

Recombinant human albumin has now been shown to be as effective as blood derived albumin in supporting fertilization [92] and embryo development, and its efficacy has been proven in a prospective randomized trial [93]. Significantly, embryos cultured in the presence of recombinant albumin exhibit an increased tolerance to cryopreservation [94]. Historically its clinical use has been restricted by price, however, with costs of such recombinant product falling, it is appropriate to re-evaluate its clinical use.

A further macromolecule present in the female reproductive tract is hyaluronan, which in the human and mouse uterus increases at the time of implantation [95, 96]. Hyaluronan is a high molecular mass polysaccharide that can be obtained endotoxin- and prion-free from a yeast fermentation procedure. Not only can hyaluronan improve mouse and bovine embryo culture systems [97, 98], but its use for embryo transfer results in a significant increase in embryo implantation [97, 99, 100]. In the largest prospective trial to date, which enrolled 1282 cycles of IVF, it was determined that the use of hyaluronan enriched medium was associated with significant increases in clinical pregnancy rates and implantation rates, both for day 3 and day 5 embryo transfers. The beneficial effect was most evident in women who were >35 years of age, in women who had only poor-quality embryos available for transfer, and in women who had previous implantation failures [100, 101]. A recent Cochrane report on 21 studies reported improved pregnancy and take-home baby rates when hyaluronan is included in the transfer medium [102], and a further study by Adeniyi and colleagues, analysing more than 3000 transfers, also concluded that the presence of high levels of hyaluronan in the transfer medium improved live birth events [103].

Of note, another highly beneficial effect of the inclusion of hyaluronan in the culture medium is on the cryo-survival of cultured embryos, data being obtained from a number of species including the human, mouse, sheep and cow [94, 99, 104–106]. As IVF programs are moving to transfer fewer embryos, there is an increasing need to be able to cryopreserve supernumerary embryos. The ability of a culture system to increase an embryo's ability to survive cryopreservation, thereby increasing cumulative pregnancy outcomes, is an important factor in deciding which culture system to use in the laboratory.

A cautionary tale of working *in vitro*

Even though the formulations of embryo culture media have improved significantly over the years, and for the most part have become more physiological in their basis, there is nothing physiological about a static drop of medium in a polystyrene culture dish or well. Therefore, one has to be careful about *in vitro* artefacts created by a static environment. A good example of this is the production of ammonium by both embryo metabolism of amino acids [107] and by the spontaneous breakdown of amino acids in the culture medium once incubated at 37°C (Figure 15.3a) [62]. Amino acids are increasingly used by human embryos as development proceeds [108], and by the blastocyst stage it produces around 25 pmol/embryo/h of ammonium [107] which can accumulate in the surrounding medium. Furthermore, the spontaneous breakdown of amino acids at 37°C contributes to the overall ammonium present in the medium. Ammonium build up in culture medium not only has negative effects on embryo development and differentiation in culture [62, 68, 109], but can affect subsequent fetal growth rates and normality at a concentration of around 300µM [33, 110]. Furthermore, it has been shown that ammonium affects embryo metabolism, pH regulation and gene expression in both the mouse and human [111–113], and that

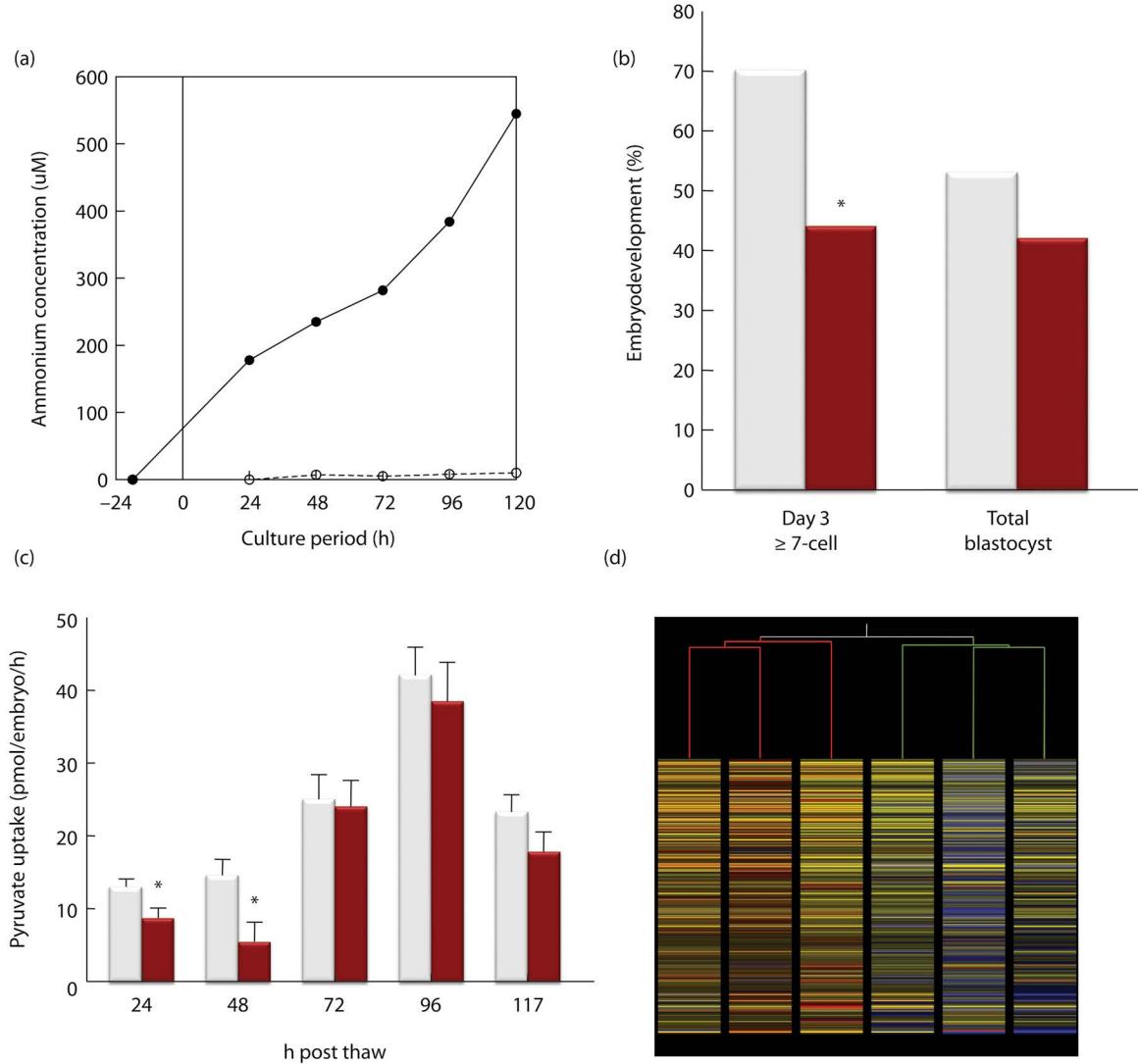


FIGURE 15.3 (a) Production of ammonium into the culture medium (lacking embryos) by the spontaneous breakdown of amino acids in culture media. Solid circles, KSOM^{AA}; Open circles, G1/G2. The media were placed in the incubator at 4 pm the day before culture for equilibration purposes. The line at time zero represents when embryos would be placed into culture (although these measurements were taken in the absence of embryos). Medium KSOM^{AA} contains 1 mM glutamine and therefore releases significant levels of ammonium into the culture medium. Media G1/G2 do not contain glutamine, but rather the stable dipeptide form, alanyl-glutamine, and therefore these media do not release significant levels of ammonium. At a concentration of just 75 $\mu\text{mol/l}$ ammonium can induce a 24-hour developmental delay in mouse fetal development by day 15 and induces the neural tube defect exencephaly in 20% of all fetuses [33]. (b) Ammonium significantly reduces the development of the cleavage stage human embryo. Pronucleate oocytes were exposed to an increasing ammonium gradient as depicted in 3a. Control media (open bars), presence of ammonium (red bars); significantly different from no ammonium, $P < 0.05$. (From [112].) (c) Ammonium significantly compromises human embryo metabolism. Pyruvate uptake was significantly reduced by ammonium at 24 and 48 hours of culture. Control media (open bars), presence of ammonium (red bars); significantly different from no ammonium, $P < 0.05$. (From [112].) (d) Ammonium significantly impairs human blastocyst gene expression. Heat map representation with hierarchical clustering of altered genes in human blastocysts following ammonium exposure and separation of control (green lines) and three ammonium (red lines) samples into distinct branches. Gene expression is related to colour, with red representing the highest levels of gene up-regulation and blue down-regulation. (From [112].)

perturbations induced by ammonium are further compromised by its interaction with atmospheric oxygen (discussed in more detail later) [114].

There is growing data on the appearance of ammonium in the culture medium over time [62, 109, 115, 116] and its toxicity to embryos, including the human [109, 112, 117]. Of relevance, an analysis of the impact of culture media composition on the live

birth rates and subsequent development of the children conceived has been reported by Dumoulin and colleagues [118, 119]. In their studies the effects of two commercial media were analysed in a day 2 transfer program, and it was determined that difference existed in embryo growth kinetics, and subsequent birth weight, which persisted through the first two years of life. Of relevance to this discussion is that one of the two media used contained free

glutamine, and hence in this group the embryos (which exhibited the growth delay) were exposed to a level of ammonium documented to adversely affect human embryo development and physiology (Figure 15.3b–d).

As amino acids are such important regulators of embryo development it is essential to alleviate this *in vitro*-created problem. An immediate answer is to renew the culture medium every 48 hours, thereby controlling ammonium concentration within non-toxic limits. A second, and complimentary, solution is to replace the most labile amino acid, glutamine, with a dipeptide form such as alanyl-glutamine. This dipeptide has the advantage of not breaking down at 37°C. Therefore, media containing this stable form of glutamine produce significantly lower levels of ammonium. Further, the overall levels of amino acids can be titrated down to an effective concentration.

Monoculture or sequential media: One size fits all or a tailored approach?

It was established in the 1960s that it was feasible to culture the 1-cell mouse embryo to the blastocyst stage in medium lacking amino acids. In the intervening decades, it has become apparent that amino acids have a significant role to play during embryo development (discussed earlier), and that medium ideally should be renewed/replenished at least every 48 hours to ensure minimal accumulation of embryo-toxic ammonium. Subsequently, all culture systems have become, by default, dynamic [50]. From a practical point of view, therefore, the amount of work and embryo manipulations required are the same whether one is working with sequential media or a monophasic system (i.e. one medium formulation for the entire pre-implantation period).

However, the two approaches to embryo culture do have some fundamental differences. Specifically, monoculture is based on the principle of letting the embryo choose what it wants during development. In contrast, sequential media were developed to accommodate the dynamics of embryo nutrition and to mirror the environment of the female reproductive tract in which the embryos is exposed to a gradient of nutrients as it passes along the oviduct into the uterus [29, 55, 56]. The significance of these nutrient gradients to the embryo in culture warrants further research as existing data on the mouse indicates that such gradients *in vitro* do impact embryo viability following transfer. For example, when the mouse zygote is cultured to the 8-cell stage and then transferred, embryo viability is highest after exposure of the embryo to a high lactate concentration (>20 mM D/L lactate), while when the embryo is cultured post-compaction to the blastocyst stage, viability is highest after exposure to lower levels of lactate (<5 mM D/L lactate) [120]. These data, support the hypothesis that the physiology of the developing conceptus is regulated by the relative concentrations of nutrients available at specific stages of development [121].

With the advent of time-lapse microscopy, we have seen the emergence of media designed specifically for the purpose of uninterrupted culture, with the aim of minimizing the build-up of ammonium [122]. These media have been shown to be effective, but further work is warranted in this area, such as the development of sequential media *in situ*, upon which existing media drops are not necessarily renewed but supplemented with a second formulation to give rise to a modified culture environment during for the post-compaction stages. Further, should embryo culture be extended beyond day 5, it is strongly recommended to renew the culture medium given the high metabolic rate of the

blastocyst and therefore its capacity to greatly reduce the levels of available nutrients within the surrounding culture medium.

How far behind embryo development in vivo is development in vitro?

Historically, embryos cultured *in vitro* lag behind their *in vivo* developed counterparts [123, 124]. However, with the development of sequential media based on the premise of meeting the changing requirements of the embryo and minimizing trauma coupled with use of reduced oxygen concentrations in the gas phase, *in vivo* rates of embryo development can now be attained *in vitro* in the mouse [4, 49, 125]. The one proviso is that each laboratory must have sufficient quality systems in place to ensure the optimum operation of a given culture system. Such advances in culture systems represent a significant development for the laboratory, for now there exists a means of producing blastocysts at the same time and with the same cell number and allocation to the inner cell mass as embryos developed in the female tract [1, 49]. Using culture media in a highly controlled environment, as detailed throughout this book, it is possible to attain high rates of human embryo development to the blastocyst stage. Using an oocyte donor model to evaluate the efficacy of culture approaches, where the age of the oocytes is typically under 30 years, it is possible not only to obtain a blastocyst formation rate of 65%, but the resultant viability (as determined by fetal heart beat following transfer) is >65% (Table 15.3). As such, oocyte donors represent as close to a human “gold standard” as one can have in an infertility clinic. With this in mind, ensuring one can attain blastocyst development of greater than 50% and implantation rates of over 50% when using donated oocytes is a good potential starting point for introducing blastocyst culture clinically, or if oocyte donation is not offered, then patients under 35 years of age could be used when analysing laboratory KPIs and transfer outcomes.

Culture systems

Several key components of the culture system are reviewed here, all of which need to be optimized as all directly impact upon media performance.

Incubation chamber

Whatever incubation chamber is chosen, a key to successful embryo culture is to minimize perturbations in the atmosphere around the embryo. The two key perturbations to avoid are pH and temperature changes. This means that ideally the environment in which the embryo is placed is not disturbed during the culture period. Practically this is difficult to achieve in a busy

TABLE 15.3 Viability of Human Embryos Conceived *In Vitro* Using an Oocyte Donor Model

Mean blastocyst development (%)	65.1
Mean number of blastocysts transferred	2.05
Mean age of recipient	40.3
Fetal Heart (per blastocyst transferred) (%)	68.0
Clinical Pregnancy Rate (per retrieval) (%)	85.2
Twins (%)	59.9

Note: All pronucleate oocytes were cultured for 48h in medium G1 at 5% O₂, 6% CO₂, and 89% N₂. On day 3 of development embryos were washed and transferred into medium G2 under same gaseous environment. Embryos were cultured in groups of 4 in 50 µl drops of medium under Ovoil (Vitrolife AB, Sweden) in 60 mm Falcon Primaria dishes. All embryos were transferred on day 5 of development. n = 950 patients [50].

clinical laboratory. The use of an individual incubation chamber, such as a modular incubator chamber or glass desiccator (such as that used to grow Louise Brown), which can be purged with the appropriate gas mix, can alleviate such concerns [77]. Using such incubator chambers, each patient's embryos can be completely isolated within an incubator, the gas phase and for the most part temperature, being unaffected when the incubator door is opened. We like to consider such chambers as "a womb with a view." However, a downside of this approach is that only three modular chambers can be placed in one incubator, thereby necessitating the acquisition of sufficient incubators. An alternative to the use of modular chambers is the use of inner doors within an incubator to significantly reduce fluctuations in the gaseous environment upon opening the incubator door. Several incubator manufacturers make incubators with inner doors. A more recent move has been the production of incubators with a greatly reduced working volume, such that rather than two double stacks of conventional incubators (giving four working chambers), one can now have three rows of smaller incubators, stacked three high, giving a total of nine chambers. This approach allows the successful allocation of one chamber to just one or two patients, thereby stabilizing the culture environment.

Incubators with infrared (IR) as opposed to thermocouple (TC) CO₂ sensors are quicker at regulating the internal environment of the chamber and are less sensitive to environmental factors and subsequently are better able to maintain a constant CO₂ level in the incubator. Therefore, incubators equipped with IR sensors will provide a more stable environment for embryo development. With regards to temperature changes, incubators with an air jacket are less susceptible to large temperature fluctuations as those with a water jacket. Again, the use of inner doors will aid in minimizing environmental fluctuations within the chamber.

Alternatives to "classic" tissue culture incubators are mini benchtop incubators which allow for direct heat transfer between the chamber and culture vessel. Such chambers also allow for a direct flow of pre-mixed gas and therefore minimize changes in pH. More recently such chambers have seen the inclusion of time-lapse capability, facilitating the constant monitoring of embryos without the need to remove them from their culture environment. Consequently, this approach has been shown to have inherent advantages for embryo development, by minimizing handling and variations in temperature and pH [3, 126].

What is evident is that it is imperative to have sufficient numbers of incubator chambers to match the caseload. This is especially true when performing extended culture. It is important to consider the number of times an incubator will be opened in a day and to keep this to a minimum. Further, it is advisable to have separate incubators for media equilibration and for embryo culture, thereby minimizing the amount of access to incubators containing embryos.

pH and carbon dioxide

When discussing pH it is noteworthy that the pHi of the embryo is around 7.2 [38, 127, 128], whereas the pH of the media routinely range from 7.25 to 7.4. Specific media components, such as lactic and amino acids directly affect and buffer pHi respectively. Of the two isomers of lactate, D- and L-, only the L form is biologically active. However, both the D- and L-forms decrease pHi of the embryo [38]. Therefore, it is advisable to use only the L-isomer of lactate and not a medium containing both the D and L forms. While high concentrations of lactate in the culture medium can

drive pHi down [38], amino acids increase the intracellular buffering capacity and help maintain the pHi at around 7.2 [37]. As the embryo has to maintain pHi against a gradient when incubated at pH 7.4, it would seem prudent to culture embryos at lower pH. However, the pH of a CO₂/bicarbonate buffered medium is not easy to quantitate. A pH electrode can be used, but one must be quick, and the same technician must take all readings to ensure consistency. A preferred and more accurate approach is to take samples of medium and measure the pH with a blood-gas analyser. A final method necessitates the presence of phenol red in the culture medium and the use of Sorensen's phosphate buffer standards. This method allows visual inspection of a medium's pH with a tube in the incubator and is accurate to 0.2 pH units [43, 45].

When using bicarbonate buffered media, the concentration of CO₂ has a direct impact on medium pH [43]. Although most media work over a wide range of pH (7.2 to 7.4), it is preferable to ensure that pH does not go over 7.4. Therefore, it is advisable to use a CO₂ concentration of between 6% and 7% to yield a medium pH of around 7.3. The amount of CO₂ in the incubation chamber can be calibrated with a Fyrite, although such an approach is only accurate to $\pm 1\%$. A more suitable method is to use a hand-held IR metering system which can be calibrated and are accurate to around 0.2%.

When using a CO₂/bicarbonate buffered medium it is essential to minimize the amount of time the culture dish is out of a CO₂ environment to prevent increases in pH. To facilitate this modified paediatric isolettes designed to maintain temperature, humidity and CO₂ concentration can be used. However, should it not be feasible to use an isolette, then the media used can be buffered with either 20–23 mM HEPES [129] or MOPS [130] together with 5–2 mM bicarbonate [131]. Such buffering systems have been shown to work effectively on their own and also in combination [132], and do not require a CO₂ environment to maintain pH. Further, an oil overlay reduces the speed of CO₂ loss and the associated increase in pH.

Oxygen

Atmospheric oxygen is ~20% and very few tissues in the body ever see such high levels. The concentration of oxygen in the lumen of the rabbit oviduct is reported to be 2%–6% [133, 134] whereas the oxygen concentration in the oviduct of hamster, rabbit, and rhesus monkey is ~8% [135]. Interestingly, the oxygen concentration in the uterus is lower than in the oviduct, ranging from 5% in the hamster and rabbit to 1.5% in the rhesus monkey [135].

Importantly, it has been demonstrated that optimum embryo development of all non-human mammalian species occurs at an oxygen concentration below 10% [85, 136, 137]. The fact that human embryos can grow at atmospheric oxygen concentration (~20%), and give rise to viable pregnancies, has led to some confusion regarding the optimal concentration for embryo culture. Consequently, the validity of having to use a reduced oxygen concentration for human embryo culture is continually challenged. The continued use of 20% oxygen in a human IVF culture system is a good example of something that has been used for over four decades and does give pregnancies; however, the question remains, does 20% oxygen adversely affect the physiology of the developing embryo before implantation?

It was initially established in the mouse model that 20% oxygen impacts embryo development as early as the first cleavage [138]. Of great interest, it was determined that 20% oxygen is detrimental to embryo development at all stages, but with the

greatest detrimental effects imparted at the cleavage stages [138]. These findings have now been evaluated clinically, and it has been determined that 20% oxygen reduces developmental rates and delays completion of the third cell cycle [139], indicating a heightened sensitivity to oxidative stress during the cleavage stages. Furthermore, it has been established in animal models that embryos cultured to the blastocyst stage in the presence of 20% oxygen have altered gene expression and perturbed proteome compared to embryos developed *in vivo* [28, 140–142]. In contrast, culture in 5% oxygen had significantly less effect on both embryonic gene expression and proteome. Similarly, 20% oxygen has been shown to adversely affect embryonic metabolism [5]. Recent data has revealed that not only does 20% oxygen compromise the utilization of both carbohydrates and amino acids throughout the preimplantation period [113], but that atmospheric oxygen also impairs the ability of the embryo to regulate against an ammonium stress [114]. Therefore, not only does oxygen induce its own trauma on the embryo, but it also increases the embryo's susceptibility to other stressors present in the culture system or laboratory [3]. Furthermore, atmospheric oxygen has recently been linked to changes to the embryonic epigenome (reviewed by [2, 3, 143]).

In support of the utilization of physiological levels of oxygen in human embryo culture, clinical data including a randomized controlled trial, support this move to more physiological conditions with lower oxygen concentrations increasing both implantation and live birth rates [144–147]. A more recent analysis on the effects of oxygen concentration on cumulative pregnancy rates has determined that 5% oxygen is associated with significantly improved outcomes compared to 20% oxygen [148].

However, in spite of the animal and clinical data describing the detrimental effects of atmospheric oxygen, it has been reported in an on-line survey, in which 265 clinics from 54 different countries participated, that <25% of IVF human embryo culture is performed exclusively under physiological (~5%) oxygen [149]. Although this survey represents only a small fraction of the world's IVF clinics, what is notable from an extensive literature review is a clear geographic difference with regard to the use of 5% oxygen. Hopefully, since the publication of the Christianson paper, fewer clinics now used atmospheric oxygen. I presented a case for the clinical introduction of physiological oxygen in human IVF over 30 years ago [43]. In the intervening decades the rationale for the discounting of atmospheric oxygen has become compelling. Consequently, the time has come to confine the use of 20% oxygen to the annals of human IVF and to ensure physiological levels of oxygen are used by all human IVF clinics [143]. It is unethical to do otherwise.

Osmolality

Ions in any medium make the largest single contribution to osmotic pressure [48]. Mouse [150] and hamster [151] embryos will develop in a wide range of osmolalities (200 to 350 mOsmols). Although conventional embryo culture media has an osmolality of between 275 and 295 mOsmols, enhanced development of mouse embryos appears to occur at reduced osmolalities [152, 153]. However, such studies were performed using simple embryo culture media, i.e. balanced salt solutions, in the absence of amino acids. It is now evident that the inclusion of osmolytes, such as betaine, or specific amino acids, such as glycine, in the culture medium can reduce any osmotic stress [5, 66, 154–157], thereby allowing apparently normal embryo development to

occur over a wider range of osmotic pressures and ion concentrations. In spite of this, paying attention to the medium osmolality is an important variable to include in the quality assurance of the IVF laboratory, and it is essential to monitor the culture system to ensure that the osmolality of the medium used does not exceed ~300 mOsmols. To ensure the osmolality of any given culture medium, it is important to use an oil overlay and to cover the drops of medium as quickly as possible. Whenever possible, prepare only one dish at a time and use cold medium to minimize evaporation and consequent increase in osmolality. This is especially important when working in a laminar flow hood, as moving air across the dish as it is being prepared will further increase media evaporation. For incubators run without humidity, as is the case for some time-lapse incubation systems, osmolality increases have been reported [158], but the effect is dependent upon several variables including the types and volume of oil used for the medium overlay, drop size, and culture dish design [159].

Incubation volumes and embryo density

Within the lumen of the female reproductive tract the developing embryo is exposed to microlitre volumes of fluid [160]. In contrast, the embryo grown *in vitro* is subject to relatively large volumes of medium of up to 1 ml [161]. Consequently, any autocrine factor(s) produced by the developing embryo will be diluted and may therefore become ineffectual. It has been demonstrated in the mouse that blastocyst formation and cell number increase when embryos are grown in groups (up to 10) or reduced volumes (around 20 µl) [162–165]. Similar results have been obtained with sheep [68] and cow embryos [166, 167], and more recently positive effects of culturing embryos in groups has been reported for the human [168], although other clinical studies failed to observe any effects [169]. Consequently, it does appear that the preimplantation mammalian embryo produces a factor(s) capable of stimulating development of both itself and surrounding embryos (Figure 15.4) [170]. Interestingly, it has been reported that during individual culture atmospheric oxygen impairs mouse embryo development, with delays during early cleavage culminating in a resultant decrease in both inner cell mass and trophectoderm development in the blastocyst [171, 172]. When 5% oxygen was used for individual embryo cultures, development was significantly improved. The negative impacts of individual culture were further improved when embryos were culture in microwells (such as those for time-lapse incubation), as opposed to drops of medium under oil [173]. While high pregnancy rates are obtained following single embryo cultures in time-lapse systems run with 5% oxygen, future studies may still confirm the best design of microwells for human embryo development [174, 175].

What day should embryo transfer be performed?

In the early days of human IVF embryos were transferred between days 1 and 3 at either the pronucleate or cleavage stages. The reason for this stems primarily from the inability of past culture systems to support the development of viable blastocysts at acceptable rates. However, with the advent of physiological culture media [6] it became feasible to perform day 5 blastocyst transfers as a matter of routine in an IVF clinic [176, 177]. This now facilitates an answer to the question: On which day of embryo development should embryos be transferred? Before answering this question, the potential advantages and disadvantages of blastocyst culture and transfer are considered.

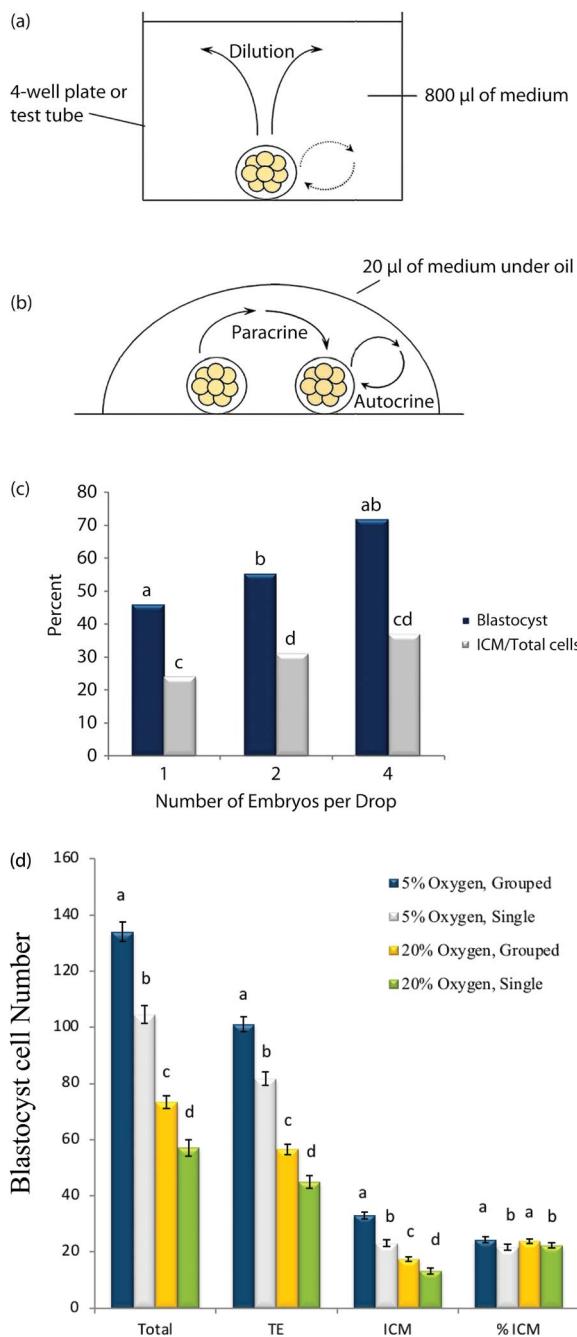


FIGURE 15.4 Effect of incubation volume and embryo grouping on embryo development and differentiation. (a) A single embryo cultured in a four-well plate or test tube, any factor produced by the embryo will become ineffectual as a result of dilution. (b) Culture of embryos in reduced volumes and/or groups increases the effective concentration of embryo-derived factors, facilitating their action in either a paracrine or autocrine manner. (c) Effect of embryo grouping on bovine blastocyst development and differentiation. Bovine embryos were cultured either individually or in groups of two or four in 50 μ l drops of medium. Like pairs are significantly different ($P < 0.05$). (Data from [167].) (d) Cell numbers on day 5 of mouse embryo culture in 5% or 20% oxygen, in groups of 10 or individually. Different letters represent significant differences between treatments; Total, TE, ICM < 0.001 , %ICM < 0.05 . (Data from [171].)

Blastocyst transfer: Advantages and disadvantages

The potential advantages of blastocyst culture and transfer have been well documented [178–181]. Advantages include:

1. Synchronizing embryonic stage with the female tract. This is important as the levels of nutrients within the fallopian tube and uterus do differ, and therefore the premature transfer of the cleavage stage embryo to the uterus could result in metabolic stress [4]. Asynchronous transfer of the cleavage stage embryo to the uterus (as opposed to the oviduct), is documented to result in poor transfer outcomes and compromised fetal development in laboratory and domestic animals [182, 183]. Furthermore, the uterine environment during a stimulated cycle cannot be considered normal. Certainly, it is known from animal studies that the hyperstimulated female tract is a less than optimal environment for the developing embryo, resulting in impaired embryo and fetal development [184–186]. Therefore, it would seem prudent to shorten the length of time an embryo is exposed to such an environment before implantation.
2. When embryos are selected for transfer at the 2- to 8-cell stage the embryonic genome has only just begun to be transcribed [36, 187], and therefore it is not possible to identify from within a given cohort those embryos with the highest developmental potential. Only by culturing embryos past the maternal/embryonic genome transition and up to the blastocyst does it become realistic to identify those embryos with limited or no developmental potential. Assessment of embryos at either the pronucleate oocyte or cleavage stages is best considered an assessment of the oocyte. Although the quality of the oocyte is important, as the quality of the developing embryo is ultimately dependent on the quality of gametes from which it is derived, it provides limited information regarding true embryo developmental potential and eliminates the impact of the male gamete on development.
3. Not all fertilized oocytes are normal, and therefore a percentage always exists that is not destined to establish a pregnancy or go to term. Factors contributing to embryonic attrition include an insufficiency of stored oocyte coded gene products, and a failure to activate the embryonic genome [188]. The culmination of this is that many abnormal embryos arrest during development *in vitro*. So, by culturing embryos to the blastocyst stage, one has already selected against those embryos with little if any developmental potential. Chromosomally abnormal human embryos can reach the blastocyst stage *in vitro* [189], and so even though euploid embryos are more likely to form blastocysts than their aneuploid siblings [190], and the frequencies of aneuploidy are significantly less at the blastocyst stage compared with cleavage stage embryo [191] blastocyst culture alone cannot be used as the sole means in identifying chromosomally abnormal embryos.
4. Uterine contractions have been negatively correlated with embryo transfer outcome, possibly by the expulsion of embryos from the uterine cavity [192]. Uterine junctional zone contractions have been quantitated and found to be strongest on the day of oocyte retrieval [193]. All patients exhibited such contractions on day 2 and 3 after retrieval, but contractility decreased and was barely evident on day 4. It is therefore feasible that the transfer of blastocysts on day 5 is, by default, associated with reduced uterine contractions and therefore there is less chance for embryonic expulsion and loss [194].

5. Trophectoderm biopsy and analysis enables the removal of more cells compared to cleavage stage embryos which facilitates the use of newer technologies such as next generation sequencing [195, 196]. It has been reported that cleavage stage biopsy for PGT actually negatively impact cycle outcomes [197], and that trophectoderm biopsy is less invasive than cleavage stage embryos for preimplantation genetic screening [198].

The potential disadvantage of extended embryo culture in a program where only blastocyst culture and transfer is offered is the possibility that a patient will not have a morula or blastocyst for transfer. Certainly there has been an increase in the percentage of patients who do not have an embryo transfer from 2.9% on day 3 to 6.7% on day 5 in one clinic [177], and from 1.3% on day 3 to 2.8% on day 5 in another [176]. Interestingly, in spite of the increase in patients not having an embryo transfer, there was a significant increase in pregnancy rate per retrieval with blastocyst culture, due to a significant increase in implantation rates.

There is significant evidence to show that in many laboratories blastocyst transfer can be more successful than cleavage stage transfer [9, 199–201]. A model previously developed to determine which patients should have SET, showed that pregnancy outcome was more favourable with day 5 than day 3 transfer [202]. Along with the published prospective randomized trials, there are retrospective studies that have concluded that day 5 transfer exhibits significant benefits for human ART in both non-selected and specific patient populations [176, 177, 203]. However, this has not been universal, and differences between outcomes likely due to the interactions of the components that have already been considered, from ovarian stimulation, culture media and system, oxygen levels, training levels, and numbers of embryologists, along with quality control.

For patients having oocyte donation, blastocyst culture and transfer is the most effective course of treatment. Oocytes from donors generally represent a more viable cohort of gametes, as they tend to come from young fertile women. Embryos derived from oocyte donors tend to reach the blastocyst stage at a higher frequency than those from IVF patients and be of higher quality. It is possible to attain an implantation rate of >65% when transferring blastocysts to recipients whose mean age is over 40 (Table 15.3) [204]. Such data not only reflect the competency of modern embryo culture systems, but emphasize the need to move to SETs, especially when performing blastocyst transfer [205].

Towards single embryo transfer

Several reviews have discussed the development of scoring systems used in clinical IVF and their significance in identifying the most viable embryo(s) for transfer [206–208] (see also Chapter 16). Certainly, with newer types of embryo culture media, implantation rates are increasing whether embryos are transferred at the cleavage stage or blastocyst. It is envisaged that for most patients, blastocyst culture and transfer will be the most effective means of being able to transfer a single embryo while maintaining high pregnancy rates, as it is evident that blastocyst score is highly predictive of implantation potential. In a prospective randomized trial of one versus two blastocysts transferred in patients with 10 or more follicles, the data indicate that it is possible to transfer a single blastocyst and obtain an ongoing pregnancy rate of 60% [205] (Figure 15.5). Subsequent trials of single blastocyst transfer versus cleavage stage embryo transfer have confirmed the higher implantation rate of the later stage embryo. It has also been established that fetal loss is significantly less following blastocyst transfer [200].

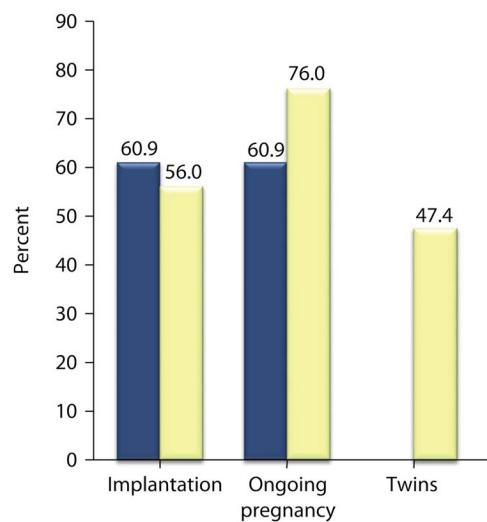


FIGURE 15.5 IVF outcome following the transfer of either one or two blastocysts. Blue bars represent the transfer of a single blastocyst (Group I), yellow bars represent the transfer of two blastocysts (Group II). Implantation and pregnancy rates were not statistically different between the two groups of patients. There were no twins in Group I in contrast to 47.4% twins in Group II. The biochemical pregnancy rate was equivalent between the two groups (Group I, 12.5%; Group II, 5%). (From [205], with permission.)

Cumulative pregnancy rates per retrieval: The significance of cryopreservation

The introduction of blastocyst culture was met with much speculation as not all laboratories were able to cryopreserve blastocysts that were not transferred. However, clinical data following blastocyst vitrification are encouraging. It has now been demonstrated that the move to blastocyst vitrification is associated with a significant increase in clinical pregnancy (50% increase) and live birth rates (40% increase) compared with those obtained with slow freezing [209]. Consequently, cumulative pregnancy data for cleavage- and blastocyst-stage embryos must be re-examined and be based upon cycles where vitrified blastocysts were utilized [143]. The latter has been reported to result in pregnancy rates and outcomes equivalent to, or even greater than, fresh transferred blastocysts [209–211]. Furthermore, the ability of a given culture system to support embryo cryo-survival is of utmost significance, with media containing hyaluronan conferring great advantage in this regard [50, 104].

Practical aspects of embryo culture

In the earlier editions of this Textbook, a section on how to prepare culture dishes was included. However, with the move to more tailor-made devices to incubate embryos, it is beyond the scope of this text to consider all dish preparation techniques which are now clearly specified by each device manufacturer. However, the basic principles outlined earlier in this chapter apply, specifically with regards to being mindful of media evaporation during dish set up, and the need to prepare a minimum number of dishes at a time. Furthermore, care must be paid to the temperature of all heated stages, the temperatures of which need to be calibrated under conditions of use, i.e. the temperature needs to be measured in the medium drop under oil, and not the actual surface of the heating stage as temperature is lost between the actual stage and the inside of the dish. Be mindful that different dish designs can affect temperature readings

depending on whether they have a lip on the bottom of the dish as this can create an air pocket and will therefore be at a different temperature to a dish whose bottom side is in direct contact with the stage. Finally, it is important to ensure accurate and gentle pipetting of the gametes and embryos themselves. It is essential to move embryos in the smallest possible volumes, made possible by using a pipette whose internal diameter is just slightly larger than the embryonic stage (never use a pipette whose inner diameter constricts the embryo). Pipetting should be slow and at no time should the embryo be moved rapidly up into a pipette, as this will increase shear stress which can have an adverse effect on development [212].

Quality control

The type of quality control used in media preparation is an important consideration when choosing a supplier, and in order for the reader to make informed decisions with regards to the types of testing offered by media providers. Establishing an appropriate quality control system for the IVF laboratory is a prerequisite in the establishment of a successful laboratory (Chapter 2). The types of bioassays conducted for this have been the focus of much discussion [213]. In reality there is no perfect model for the human, save for the very patients we treat. Consequently, it is important to understand the limitations of the assays performed and to use data obtained from bioassays in an appropriate fashion. Quality control should not be limited to the culture media used but should include all contact supplies and gases used in an IVF procedure. The bioassay favoured is the culture of pronucleate mouse oocytes in protein-free media. There has been a lot of conflicting data regarding the use of the mouse embryo assay (MEA), but by adjusting conditions, one can not only increase the sensitivity of the assay but can also quantitate quality with it.

First of all, when using the MEA, the stage at which the embryo is cultured from has an impact on development. Mouse embryos collected at the pronucleate stage do not tend to fair as well in culture as those collected at the 2-cell stage, consistent with their heightened sensitivity to stress. Second, the strain of mice is important. Embryos from hybrid parents have a decided advantage in culture, and do not represent the diverse genetic background one is dealing with in an infertility clinic. Therefore, a random bred strain of mice provides greater genetic diversity [214]. Third, the embryo cultures should be performed in the absence of protein, as protein has the ability to mask the effects of any potential toxins present. Reports that mouse embryos can develop in culture in medium prepared using tap water [215, 216] should be interpreted carefully after considering the strain of mouse, types of media used and the supplementation of medium with protein. Silverman et al. [215] used Ham's F-10. This medium contains amino acids, which can chelate any possible toxins present in the tap water, e.g. heavy metals. George et al. [216], included high levels of BSA in their zygote cultures to the blastocyst. Albumin can chelate potential embryo-toxins and thereby mask the effect of any present in the culture medium [217, 218]. Furthermore, all such studies used blastocyst development as the sole criterion for assessing embryo development. Blastocyst development is a poor indicator of embryo quality and does not accurately reflect developmental potential [74]. Therefore, rates of development should be determined by scoring the embryos at specific times during culture. Key times to examine the embryos include the morning of day 3 to determine the extent of compaction, the afternoon of day 4 to determine the

degree of blastocyst formation and the morning of day 5 to assess the initiation of hatching [213]. This latter approach can now be readily applied through the utilization of time-lapse microscopy [219].

Finally, the embryos that form blastocysts in a given time, typically on the morning of day 5, should have their cell numbers determined, as blastocyst cell number is a good indicator of subsequent development potential. When new components of certain culture media could affect the development of the inner cell mass directly, such as essential amino acids, a differential nuclear stain should be performed in order to determine the extent of ICM development. Using such an approach it is possible to identify potential problems in culture media before they are used clinically. In our experience around 25% of all contact supplies fail such pre-screening [213]. Although some of the contact supplies that fail the bioassay are not outright lethal, they do compromise embryo development. If undetected this would result in reduced clinical pregnancy rates. Consequently, this helps to explain periodic changes in clinical pregnancy rates and emphasizes the significance of an ongoing quality control program. The majority of products on the market are now pre-screened for embryo toxicity. However, it is worth noting that not all testing is the same and that it is worth understanding the sensitivity of the assay used before introduction of an item into the laboratory. However, irrespective of the testing, all supplies should be tracked as they enter the laboratory to confirm efficacy for human embryos.

Future developments in embryo culture systems

Antioxidants

A subject already touched upon in this chapter is the toxicity of oxygen, induced in part through the induction of reactive oxygen species. As a result of the growing data on the pathologies induced by atmospheric oxygen, there has been a resurgence of interest in the role of antioxidants in supporting IVF and embryo development (reviewed by Gardner and Truong [220]). Rather than focusing on individual antioxidants, recent works have concentrated on the efficacy of groups of antioxidants, a more physiologically approach. Truong and colleagues, working with the mouse model, were able to establish that a combination of alpha-lipoic acid (5 uM), acetyl-carnitine (10 uM) and N-acetyl-L-cysteine (10 uM) was highly effective at protecting the developing embryo from oxidative stress at both 5% and 20% oxygen, culminating in blastocyst cell numbers and an increase in viability post transfer [172]. Subsequent studies determined that this group of three antioxidants was also effective at increasing both IVF and vitrification outcomes [221, 222], and that the gene expression of the resultant fetuses and placentae was closer to *in vivo* developed controls when antioxidants were present for embryo culture and for vitrification [142]. These three antioxidants were then evaluated in a randomized sibling oocyte study, in which it was shown that ongoing pregnancies were increased in patients 35 to 40 years of age [223]. Subsequent larger clinical studies will help to establish the roles of antioxidants in human ART.

Growth factors and cytokines

Growth factors and cytokines are present in the fluids of the human female reproductive tract, with increasing abundance in

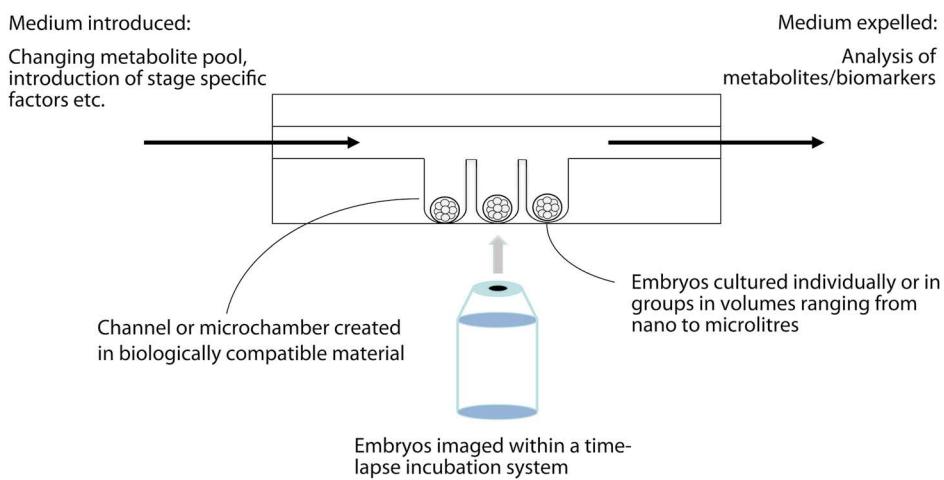


FIGURE 15.6 Schematic of an embryo perfusion culture system. Culture media are continuously passed over the embryo(s). The composition of the culture media can be changed according to the specific requirements of each stage of embryonic development. Toxins such as ammonium are not able to build up and impair embryo development, while more labile components of the culture system are not denatured. Further, media can be sampled in real time to quantitate embryo physiology. (Modified from [40].)

the uterus [224, 225], and have been shown to have effects on animal embryo viability [226], and yet they are conspicuously absent from clinical embryo culture media. An exception to this is a study on the effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) [227]. However, it was reported that GM-CSF only had a beneficial effect when the levels of HSA were reduced in the medium, an observation also previously reported in the mouse model [228]. Similar to antioxidants, more recent works have focused on the efficacy of groups of growth factors, as opposed to the inclusion of individual factors [229, 230]. While it has been shown that such an approach can improve embryo development *in vitro* and subsequent fetal development, subsequent analysis of both fetal and placental gene expression following embryo transfer in the mouse, revealed aberrant transcription profiles compared to embryo developed *in vivo* [230]. Hence, although there was a positive effect in culture, the normalcy of the resultant fetuses and subsequent developmental programming following culture in the presence of growth factors and cytokines is brought into question. Further in-depth works on animal models, including the long-term follow-up of offspring, are therefore warranted before the addition of such factors to human embryo culture media can be considered safe.

Perfusion culture

As discussed previously, there is nothing physiological about the physical conditions in which embryos are cultured. Rather than a static drop of medium, the future may engage perfusion culture systems, enabling the embryo to be exposed to a flux of nutrients and factors (Figure 15.6) [40, 231, 232] (Chapter 28 by Goss et al.). This latter approach has the advantage of being able to expose embryos to numerous gradients and fresh media throughout development. Furthermore, samples of medium can be taken and analysed for carbohydrates [233], amino acids [234], and other factors related to implantation potential post

transfer [7, 39] (Chapter 16 by Sakkas and Gardner). Although this concept has been considered for several decades, the available technologies were not sufficiently developed in order for this to be evaluated. With recent advances in 3D printing and 2 photon polymerization, it is now possible to fabricate devices capable of perfusing nanolitre volumes of culture media over preimplantation embryos during their culture [235] (see Chapter 28 by Goss et al.). Consequently, in the next few years we shall be able to fully evaluate perfusion culture in human ART.

Conclusions

In this chapter the complexities of human embryo culture have been considered, and advances in culture technologies discussed. Rather than perceiving embryo culture as an optimized procedure, it should be considered as continuously improving process as we learn more about the preimplantation human embryo and its environment *in vivo* [7]. In combination with the introduction of new technologies, this will ultimately lead to greater efficacies and efficiencies while also paving the way to automation of several key laboratory processes [236]. Diligent monitoring and reporting of pregnancy outcomes and consideration of cumulative pregnancy rates per cycle will help in the continued improvement and evaluation of assisted conception. As more reports on IVF outcomes become available, it is essential that when interpreting findings careful attention is paid to precise the conditions used by clinics, given the number of variables associated with laboratory processes which can affect outcomes [237] (Table 15.4). This is especially important for oxygen concentration, which as discussed in detail, has the capacity to affect the embryo in a number of ways. Indeed 20% oxygen can compromise an otherwise safe and effective culture system, which in turn will lead to erroneous conclusions with regards to clinical outcomes [143].

TABLE 15.4 Effects of Laboratory Conditions on Human Embryo Phenotype

IVF Laboratory Conditions	Observed Effects on Human Preimplantation Embryo Phenotype
Amino acids	Increases the rate of embryo development and blastocyst formation
Ammonium	Decreases cleavage rate and metabolism; decreases blastocyst formation and alters gene expression
Carbohydrates	Stage specific response to nutrients. Gradients of key nutrients such as lactate will regulate the intracellular redox
Serum	Sequestration of lipids resulting in an altered physiology and possible effects on gene expression and imprinting
Oxygen ^a	Atmospheric oxygen decreases cleavage times, decreases blastocyst formation, reduce cell number and further it alters embryonic metabolism, the transcriptome and epigenome.
Embryo density/single culture	Affects blastocyst formation, embryo morphology and pregnancy rate
Microwells	Increase in blastocyst formation, morphology scores, and cells in ICM
Undisturbed culture	Current data contradictory
Air Quality	Filtered air increases blastocyst formation and morphology scores, and live birth rate
Oil	Poor oils reduce morphological quality on Day 3
Osmolality	Hypo-osmotic conditions induce release of osmolytes and activation of anion channels
pH	Oocytes exposed to acidic or alkaline environment are less likely to reach first cleavage and have higher fragmentation
Temperature	Fluctuations are detrimental to oocyte spindle stability and fertilization. Reduced temperature during embryo culture results in fewer cells and blastocyst formation.

Note:

- ^a Not only does atmospheric oxygen have its own direct negative effects on several key processes within the embryo, it also increases the susceptibility of the embryo to a second stressor. Examples of this include individual culture where development is significantly impaired in the presence of atmospheric oxygen, and the ability of the embryo to regulate against ammonium toxicity. In the latter case, atmospheric oxygen reduces the ability of an embryo to utilize amino acids and to transaminate ammonium.

References

- Gardner D, Lane M. Towards a single embryo transfer. *Reprod Biomed Online*. 2003;6:470–81.
- Gardner DK, Kelley RL. Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *J Dev Orig Health Dis*. 2017;8:418–35.
- Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update*. 2016;22:2–22.
- Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology*. 1998;49:83–102.
- Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev*. 2005;17:371–8.
- Gardner DK, Lane M. Culture and selection of viable blastocysts: A feasible proposition for human IVF? *Hum Reprod Update*. 1997;3:367–82.
- Gardner DK, Sakkas D. Making and selecting the best embryo in the laboratory. *Fertil Steril*. 2023.
- Papanikolaou EG, Kolibianakis EM, Tournaye H, Venetis CA, Fatemi H, Tarlatzis B, et al. Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis. *Hum Reprod*. 2008;23:91–9.
- Glujovsky D, Retamar Q, Sedo A, Ciapponi CR, Cornelisse A, Blake S. Cleavage-stage versus blastocyst-stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev*. 2022;5:CD002118.
- Fiorentino F, Bono S, Biricik A, Nuccitelli A, Cotroneo E, Cottone G, et al. Application of next-generation sequencing technology for comprehensive aneuploidy screening of blastocysts in clinical preimplantation genetic screening cycles. *Hum Reprod*. 2014;29:2802–13.
- Bergh C. Single embryo transfer: A mini-review. *Hum Reprod*. 2005;20:323–7.
- Adashi EY, Barri PN, Berkowitz R, Braude P, Bryan E, Carr J, et al. Infertility therapy-associated multiple pregnancies (births): An ongoing epidemic. *Reprod Biomed Online*. 2003;7:515–42.
- Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril*. 2013;99:1062–72.
- Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev*. 2015;27:638–54.
- Leese HJ. Metabolism of the preimplantation mammalian embryo. *Oxf Rev Reprod Biol*. 1991;13:35–72.
- Hardy K, Hooper MA, Handyside AH, Rutherford AJ, Winston RM, Leese HJ. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Hum Reprod*. 1989;4:188–91.
- Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem*. 2005;280:18361–7.
- Ratnam S, Mertineit C, Ding F, Howell CY, Clarke HJ, Bestor TH, et al. Dynamics of Dnmt1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development. *Dev Biol*. 2002;245:304–14.
- Huang JC, Lei ZL, Shi LH, Miao YL, Yang JW, Ouyang YC, et al. Comparison of histone modifications in *in vivo* and *in vitro* fertilization mouse embryos. *Biochem Biophys Res Commun*. 2007;354:77–83.
- Lucifero D, La Salle S, Bourc'his D, Martel J, Bestor TH, Trasler JM. Coordinate regulation of DNA methyltransferase expression during oogenesis. *BMC Dev Biol*. 2007;7:36.
- Dumollard R, Marangos P, Fitzharris G, Swann K, Duchen M, Carroll J. Sperm-triggered [Ca²⁺] oscillations and Ca²⁺ homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. *Development*. 2004;131:3057–67.
- Gangaraju VK, Bartholomew B. Mechanisms of ATP dependent chromatin remodeling. *Mutat Res*. 2007;618:3–17.
- Pepin D, Vanderhyden BC, Picketts DJ, Murphy BD. ISWI chromatin remodeling in ovarian somatic and germ cells: Revenge of the NURFs. *Trends Endocrinol Metab*. 2007;18:215–24.
- Harvey AJ, Rathjen J, Gardner DK. Metaboloepigenetic regulation of pluripotent stem cells. *Stem Cells Int*. 2016;2016:1816525.

25. Warburg O. On the origin of cancer cells. *Science*. 1956;123: 309–14.
26. Gardner DK. Lactate production by the mammalian blastocyst: Manipulating the microenvironment for uterine implantation and invasion? *Bioessays*. 2015;37:364–71.
27. Gurner KH, Evans J, Hutchison JC, Harvey AJ, Gardner DK. A microenvironment of high lactate and low pH created by the blastocyst promotes endometrial receptivity and implantation. *Reprod Biomed Online*. 2022;44:14–26.
28. Gardner DK, Lane M. Ex vivo early embryo development and effects on gene expression and imprinting. *Reprod Fertil Dev*. 2005;17:361.
29. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: Metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril*. 1996;65:349–53.
30. Feuer SK, Camarano L, Rinaudo PF. ART and health: Clinical outcomes and insights on molecular mechanisms from rodent studies. *Mol Hum Reprod*. 2013;19:189–204.
31. Bloise E, Feuer SK, Rinaudo PF. Comparative intrauterine development and placental function of ART concepti: Implications for human reproductive medicine and animal breeding. *Hum Reprod Update*. 2014;20:822–39.
32. Calle A, Fernandez-Gonzalez R, Ramos-Ibeas P, Laguna-Barraza R, Perez-Cerezales S, Bermejo-Alvarez P, et al. Long-term and transgenerational effects of in vitro culture on mouse embryos. *Theriogenology*. 2012;77:785–93.
33. Lane M, Gardner DK. Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil*. 1994;102:305–12.
34. Zander DL, Thompson JG, Lane M. Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol Reprod*. 2006;74: 288–94.
35. Rooke JA, McEvoy TG, Ashworth CJ, Robinson JJ, Wilmut I, Young LE, et al. Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. *Theriogenology*. 2007;67:639–47.
36. Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature*. 1988;332:459–61.
37. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: Amino acids act as buffers of intracellular pH. *Hum Reprod*. 1998;13:3441–8.
38. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: Effects of extracellular pH and weak acids. *Mol Reprod Dev*. 1998;50:434–42.
39. Gardner DK, Meseguer M, Rubio C, Treff NR. Diagnosis of human preimplantation embryo viability. *Hum Reprod Update*. 2015;21(6):727–47.
40. Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int*. 1994;18:1163–79.
41. Bavister BD. Culture of preimplantation embryos: Facts and artifacts. *Hum Reprod Update*. 1995;1:91–148.
42. Leese HJ. Metabolic control during preimplantation mammalian development. *Hum Reprod Update*. 1995;1:63–72.
43. Gardner DK, Lane M. Embryo culture systems. In: *Handbook of In Vitro Fertilization*, 2nd ed. Trounson A, Gardner DK (eds). Boca Raton, FL: CRC Press, pp. 205–64, 1999.
44. Pool TB. Recent advances in the production of viable human embryos in vitro. *Reprod Biomed Online*. 2002;4:294–302.
45. Gardner DK, Lane M. Embryo culture systems. In: *In Vitro Fertilization a Practical Approach*. Gardner DK (ed). New York, NY: Informa Healthcare, pp. 221–82, 2007.
46. Summers MC, Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: Historical perspective and current issues. *Hum Reprod Update*. 2003;9: 557–82.
47. Lane M, Gardner DK. Embryo culture medium: Which is the best? *Best Pract Res Clin Obstet Gynaecol*. 2007;21:83–100.
48. Gardner DK, Lane M. Embryo culture systems. In: *Handbook of In Vitro Fertilization*, 4th ed. Gardner DK, Simon C (eds). Boca Raton, FL: CRC Press, pp. 205–44, 2017.
49. Gardner DK, Lane M. Sequential media for human blastocyst culture. In: *In Vitro Fertilization. A Textbook of Current and Emerging Methods and Devices*, 2nd ed. Nagy ZP, Varghese AC, Agarwal A (eds). Cham, Springer Nature Switzerland, , pp. 157–70, 2019.
50. Gardner DK. Dissection of culture media for embryos: The most important and less important components and characteristics. *Reprod Fertil Dev*. 2008;20:9–18.
51. Perkins JL, Goode L. Free amino acids in the oviduct fluid of the ewe. *J Reprod Fertil*. 1967;14:309–11.
52. Casslen BG. Free amino acids in human uterine fluid. Possible role of high taurine concentration. *J Reprod Med*. 1987;32:181–4.
53. Miller JG, Schultz GA. Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biol Reprod*. 1987;36:125–9.
54. Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil*. 1990;88:361–8.
55. Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology*. 2005;64:992–1006.
56. Hugentobler SA, Diskin MG, Leese HJ, Humpherson PG, Watson T, Sreenan JM, et al. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. *Mol Reprod Dev*. 2007;74:445–54.
57. Van Winkle LJ. Amino acid transport regulation and early embryo development. *Biol Reprod*. 2001;64:1–12.
58. Schultz GA, Kaye PL, McKay DJ, Johnson MH. Endogenous amino acid pool sizes in mouse eggs and preimplantation embryos. *J Reprod Fertil*. 1981;61:387–93.
59. Gardner DK, Clarke RN, Lechene CP, Biggers JD. Development of a noninvasive ultramicrofluorometric method for measuring net uptake of glutamine by single preimplantation mouse embryos. *Gamete Res*. 1989;24:427–38.
60. Rieger D, Loskutoff NM, Betteridge KJ. Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured in vitro. *J Reprod Fertil*. 1992;95:585–95.
61. Eagle H. Amino acid metabolism in mammalian cell cultures. *Science*. 1959;130:432–7.
62. Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod*. 1993;48:377–85.
63. Dumoulin JC, Evers JL, Bakker JA, Bras M, Pieters MH, Geraedts JP. Temporal effects of taurine on mouse preimplantation development in vitro. *Hum Reprod*. 1992;7:403–7.
64. Dumoulin JC, Evers JL, Bras M, Pieters MH, Geraedts JP. Positive effect of taurine on preimplantation development of mouse embryos in vitro. *J Reprod Fertil*. 1992;94:373–80.
65. Gardner DK, Lane M. The 2-cell block in CF1 mouse embryos is associated with an increase in glycolysis and a decrease in tricarboxylic acid (TCA) cycle activity: Alleviation of the 2-cell block is associated with the restoration of in vivo metabolic pathway activities. *Biol Reprod*. 1993;49(Suppl.1):152 (Abstract).
66. Bavister BD, McKiernan SH. Regulation of hamster embryo development in vitro by amino acids. In: *Preimplantation Embryo Development*. Bavister BD (ed). New York, NY: Springer-Verlag, pp. 57–72, 1992.
67. McKiernan SH, Clayton MK, Bavister BD. Analysis of stimulatory and inhibitory amino acids for development of hamster one-cell embryos in vitro. *Mol Reprod Dev*. 1995;42:188–99.
68. Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: Amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod*. 1994;50:390–400.

69. Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biol Reprod.* 1995;53:1385–91.
70. Takahashi Y, First NL. In vitro development of bovine one-cell embryos influence of glucose, lactate, amino acids and vitamins. *Theriogenology.* 1992;37:963–78.
71. Steeves TE, Gardner DK. Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol Reprod.* 1999;61:731–40.
72. Devreker F, Winston RM, Hardy K. Glutamine improves human preimplantation development in vitro. *Fertil Steril.* 1998;69:293–9.
73. Devreker F, Van den Bergh M, Biramane J, Winston RL, Englert Y, Hardy K. Effects of taurine on human embryo development in vitro. *Hum Reprod.* 1999;14:2350–6.
74. Lane M, Gardner DK. Differential regulation of mouse embryo development and viability by amino acids. *J Reprod Fertil.* 1997;109:153–64.
75. Lane M, Gardner DK. Nonessential amino acids and glutamine decrease the time of the first three cleavage divisions and increase compaction of mouse zygotes in vitro. *J Assist Reprod Genet.* 1997;14:398–403.
76. Menezo Y, Clement P, Dale B, Elder K. Modulating oxidative stress and epigenetic homeostasis in preimplantation IVF embryos. *Zygote.* 2022;30:149–58.
77. Gardner DK, Lane M. Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: Role of amino acids, EDTA and physical parameters. *Hum Reprod.* 1996;11:2703–12.
78. Ho Y, Wigglesworth K, Eppig JJ, Schultz RM. Preimplantation development of mouse embryos in KSOM: Augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev.* 1995;41:232–8.
79. Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod.* 2000;62:1526–35.
80. Dorland M, Gardner DK, Trounson A. Serum in synthetic oviduct fluid causes mitochondrial degeneration in ovine embryos. *Reprod Fertil (Abstr Ser).* 1994;13:70.
81. Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod.* 2001;64:918–26.
82. Wrenzycki C, Herrmann D, Keskinpepe L, Martins A Jr, Sirisathien S, Brackett B, et al. Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Hum Reprod.* 2001;16:893–901.
83. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, et al. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet.* 2001;27:153–4.
84. Gray CW, Morgan PM, Kane MT. Purification of an embryotrophic factor from commercial bovine serum albumin and its identification as citrate. *J Reprod Fertil.* 1992;94:471–80.
85. Batt PA, Gardner DK, Cameron AW. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. *Reprod Fertil Dev.* 1991;3:601–7.
86. McKiernan SH, Bavister BD. Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos. *Vitro Cell Dev Biol.* 1992;28A:154–6.
87. Kane MT. Variability in different lots of commercial bovine serum albumin affects cell multiplication and hatching of rabbit blastocysts in culture. *J Reprod Fertil.* 1983;69:555–8.
88. Bar-Or D, Bar-Or R, Rael LT, Gardner DK, Slone DS, Craun ML. Heterogeneity and oxidation status of commercial human albumin preparations in clinical use. *Crit Care Med.* 2005;33:1638–41.
89. Takatori S, Akutsu K, Kondo F, Ishii R, Nakazawa H, Makino T. Di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in media for in vitro fertilization. *Chemosphere.* 2012;86:454–9.
90. Akutsu K, Takatori S, Nakazawa H, Makino T. Detection of polybrominated diphenyl ethers in culture media and protein sources used for human in vitro fertilization. *Chemosphere.* 2013;92:864–9.
91. Morbeck DE, Paczkowski M, Fredrickson JR, Krisher RL, Hoff HS, Baumann NA, et al. Composition of protein supplements used for human embryo culture. *J Assist Reprod Genet.* 2014;31:1703–11.
92. Bavister BD, Kinsey DL, Lane M, Gardner DK. Recombinant human albumin supports hamster in-vitro fertilization. *Hum Reprod.* 2003;18:113–6.
93. Bungum M, Humaidan P, Bungum L. Recombinant human albumin as protein source in culture media used for IVF: A prospective randomized study. *Reprod Biomed Online.* 2002;4:233–6.
94. Lane M, Maybach JM, Hooper K, Hasler JF, Gardner DK. Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. *Mol Reprod Dev.* 2003;64:70–8.
95. Zorn TM, Pinhal MA, Nader HB, Carvalho JJ, Abrahamsohn PA, Dietrich CP. Biosynthesis of glycosaminoglycans in the endometrium during the initial stages of pregnancy of the mouse. *Cell Mol Biol (Noisy-le-grand).* 1995;41:97–106.
96. Salamonson LA, Shuster S, Stern R. Distribution of hyaluronan in human endometrium across the menstrual cycle. Implications for implantation and menstruation. *Cell Tissue Res.* 2001;306:335–40.
97. Gardner DK, Rodriguez-Martinez H, Lane M. Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer. *Hum Reprod.* 1999;14:2575–80.
98. Palasz AT, Rodriguez-Martinez H, Beltran-Brena P, Perez-Garnelo S, Martinez MF, Gutierrez-Adan A, et al. Effects of hyaluronan, BSA, and serum on bovine embryo in vitro development, ultrastructure, and gene expression patterns. *Mol Reprod Dev.* 2006;73:1503–11.
99. Dattena M, Mara L, Bin TA, Cappai P. Lambing rate using vitrified blastocysts is improved by culture with BSA and hyaluronan. *Mol Reprod Dev.* 2007;74:42–7.
100. Urman B, Yakin K, Ata B, Isiklar A, Balaban B. Effect of hyaluronan-enriched transfer medium on implantation and pregnancy rates after day 3 and day 5 embryo transfers: A prospective randomized study. *Fertil Steril.* 2008;90:604–12.
101. Bontekoe S, Blake D, Heineman MJ, Williams EC, Johnson N. Adherence compounds in embryo transfer media for assisted reproductive technologies. *Cochrane Data Syst Rev.* 2010;CD007421.
102. Heymann D, Vidal L, Or Y, Shoham Z. Hyaluronic acid in embryo transfer media for assisted reproductive technologies. *Cochrane Data Syst Rev.* 2020;9:CD007421.
103. Adeniyi T, Horne G, Ruane PT, Brison DR, Roberts SA. Clinical efficacy of hyaluronate-containing embryo transfer medium in IVF/ICSI treatment cycles: A cohort study. *Hum Reprod Open.* 2021;2021:hoab004.
104. Balaban B, Urman B. Comparison of two sequential media for culturing cleavage-stage embryos and blastocysts: Embryo characteristics and clinical outcome. *Reprod Biomed Online.* 2005;10:485–91.
105. Stojkovic M, Kolle S, Peinl S, Stojkovic P, Zakhartchenko V, Thompson JG, et al. Effects of high concentrations of hyaluronan in culture medium on development and survival rates of fresh and frozen-thawed bovine embryos produced in vitro. *Reproduction.* 2002;124:141–53.
106. Palasz AT, Brena PB, Martinez MF, Perez-Garnelo SS, Ramirez MA, Gutierrez-Adan A, et al. Development, molecular composition and freeze tolerance of bovine embryos cultured in TCM-199 supplemented with hyaluronan. *Zygote.* 2008;16:39–47.
107. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril.* 2001;76:1175–80.

108. Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, et al. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod.* 2002;17:999–1005.
109. Virant-Klun I, Tomazevic T, Vrtacnik-Bokal E, Vogler AK, Meden-Vrtovec M. Increased ammonium in culture medium reduces the development of human embryos to the blastocyst stage. *Fertil Steril.* 2006;85:526–8.
110. Sinawat S, Hsiao WC, Flockhart JH, Kaufman MH, Keith J, West JD. Fetal abnormalities produced after preimplantation exposure of mouse embryos to ammonium chloride. *Hum Reprod.* 2003;18:2157–65.
111. Lane M, Gardner DK. Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biol Reprod.* 2003;69:1109–17.
112. Gardner DK, Hamilton R, McCallie B, Schoolcraft WB, Katz-Jaffe MG. Human and mouse embryonic development, metabolism and gene expression are altered by an ammonium gradient in vitro. *Reproduction.* 2013;146:49–61.
113. Wale PL, Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod.* 2012;87:24.
114. Wale PL, Gardner DK. Oxygen affects the ability of mouse blastocysts to regulate ammonium. *Biol Reprod.* 2013;89:75.
115. Nakazawa T, Ohashi K, Yamada M, Shinoda S, Saji F, Murata Y, et al. Effect of different concentrations of amino acids in human serum and follicular fluid on the development of one-cell mouse embryos in vitro. *J Reprod Fertil.* 1997;111:327–32.
116. Lane M, Gardner DK. Removal of embryo-toxic ammonium from the culture medium by *in situ* enzymatic conversion to glutamate. *J Exp Zool.* 1995;271:356–63.
117. Zhao H, Qiu Q, Ou S, Lin H, Wang W, Zhang Q. Increased ammonium in culture medium may promote cellular apoptosis and negatively affect pluripotency of human blastocysts. *Arch Gynecol Obstet.* 2023;307:619–624.
118. Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, et al. Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod.* 2010;25:605–12.
119. Kleijkers SH, van Montfoort AP, Smits LJ, Viechtbauer W, Roseboom TJ, Nelissen EC, et al. IVF culture medium affects post-natal weight in humans during the first 2 years of life. *Hum Reprod.* 2014;29:661–9.
120. Gardner DK, Sakkas D. Mouse embryo cleavage, metabolism and viability: Role of medium composition. *Hum Reprod.* 1993;8:288–95.
121. Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. *Biol Reprod.* 2000;62:16–22.
122. Hardarson T, Bungum M, Conaghan J, Meintjes M, Chantilis SJ, Molnar L, et al. Noninferiority, randomized, controlled trial comparing embryo development using media developed for sequential or undisturbed culture in a time-lapse setup. *Fertil Steril.* 2015;104:1452–9 e1–4.
123. Bowman P, McLaren A. Cleavage rate of mouse embryos *in vivo* and *in vitro*. *J Embryol Exp Morphol.* 1970;24:203–7.
124. Harlow GM, Quinn P. Development of preimplantation mouse embryos *in vivo* and *in vitro*. *Aust J Biol Sci.* 1982;35:187–93.
125. Gardner DK, Lane M. Development of viable mammalian embryos *in vitro*: Evolution of sequential media. In: *Principles of Cloning*. Cibelli R, Lanza K, Campbell AK, West MD (eds). San Diego: Academic Press, pp. 187–213, 2002.
126. Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, Requena A. Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: A retrospective cohort study. *Fertil Steril.* 2012;98:1481–9 e10.
127. Phillips KP, Leveille MC, Claman P, Baltz JM. Intracellular pH regulation in human preimplantation embryos. *Hum Reprod.* 2000;15:896–904.
128. Lane M, Baltz JM, Bavister BD. Regulation of intracellular pH in hamster preimplantation embryos by the sodium hydrogen (Na^+/H^+) antiporter. *Biol Reprod.* 1998;59:1483–90.
129. Quinn P, Barros C, Whittingham DG. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J Reprod Fertil.* 1982;66:161–8.
130. Lane M, Gardner DK. Preparation of gametes, *in vitro* maturation, *in vitro* fertilization, embryo recovery and transfer. In: *A Laboratory Guide to the Mammalian Embryo*. Gardner DK, Lane M, Watson AJ (eds). New York, NY: Oxford Press, pp. 24–40, 2004.
131. Gardner DK, Lane M. Mammalian preimplantation embryo culture. *Methods Mol Biol.* 2014;1092:167–82.
132. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod Biomed Online.* 2009;18:799–810.
133. Mastroianni L Jr, Jones R. Oxygen tension within the rabbit fallopian tube. *J Reprod Fertil.* 1965;147:99–102.
134. Ross RN, Graves CN. O_2 levels in female rabbit reproductive tract. *J Anim Sci.* 1974;39:994.
135. Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil.* 1993;99:673–9.
136. Quinn P, Harlow GM. The effect of oxygen on the development of preimplantation mouse embryos *in vitro*. *J Exp Zool.* 1978;206:73–80.
137. Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on *in-vitro* development of preimplantation sheep and cattle embryos. *J Reprod Fertil.* 1990;89:573–8.
138. Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients. *Reprod Biomed Online.* 2010;21:402–10.
139. Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertil Steril.* 2013;99:738–44 e4.
140. Katz-Jaffe MG, Linck DW, Schoolcraft WB, Gardner DK. A proteomic analysis of mammalian preimplantation embryonic development. *Reproduction.* 2005;130:899–905.
141. Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM. Effects of oxygen tension on gene expression in preimplantation mouse embryos. *Fertil Steril.* 2006;86:1252–65, 65 e1–36.
142. Truong T, Harvey AJ, Gardner DK. Antioxidant supplementation of mouse embryo culture or vitrification media support more *in-vivo*-like gene expression post-transfer. *Reprod Biomed Online.* 2022;44:393–410.
143. Gardner DK. The impact of physiological oxygen during culture, and vitrification for cryopreservation, on the outcome of extended culture in human IVF. *Reprod Biomed Online.* 2016;32:137–41.
144. Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, et al. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. *Hum Reprod.* 2009;24:300–7.
145. Nanassy L, Peterson CA, Wilcox AL, Peterson CM, Hammoud A, Carrell DT. Comparison of 5% and ambient oxygen during days 3–5 of *in vitro* culture of human embryos. *Fertil Steril.* 2010;93:579–85.
146. Waldenstrom U, Engstrom AB, Hellberg D, Nilsson S. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. *Fertil Steril.* 2009;91:2461–5.
147. Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Data Syst Rev.* 2012;7:CD008950.

148. Van Montfoort APA, Arts E, Wijnandts L, Sluijmer A, Pelinck MJ, Land JA, et al. Reduced oxygen concentration during human IVF culture improves embryo utilization and cumulative pregnancy rates per cycle. *Hum Reprod Open*. 2020;2020:hoz036.
149. Christianson MS, Zhao Y, Shoham G, Granot I, Safran A, Khafagy A, et al. Embryo catheter loading and embryo culture techniques: Results of a worldwide web-based survey. *J Assist Reprod Genet*. 2014;31:1029–36.
150. Brinster RL. Studies on the development of mouse embryos in vitro. I. The effect of osmolarity and hydrogen ion concentration. *J Exp Zool*. 1965;158:49–57.
151. Bavister BD, Golden M. Alteration of extracellular cation concentrations and ratios in culture medium does not affect first cleavage division of hamster zygotes in vitro nor overcome the ‘two-cell block.’ *Reprod Fertil Dev*. 1989;1:231–6.
152. Whitten WK, Biggers JD. Complete development in vitro of the pre-implantation stages of the mouse in a simple chemically defined medium. *J Reprod Fertil*. 1968;17:399–401.
153. Lawitts JA, Biggers JD. Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. *Mol Reprod Dev*. 1992;31:189–94.
154. Van Winkle LJ, Haghigiat N, Campione AL. Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. *J Exp Zool*. 1990;253:215–9.
155. Biggers JD, Lawitts JA, Lechene CP. The protective action of betaine on the deleterious effects of NaCl on preimplantation mouse embryos in vitro. *Mol Reprod Dev*. 1993;34:380–90.
156. Dawson KM, Collins JL, Baltz JM. Osmolarity-dependent glycine accumulation indicates a role for glycine as an organic osmolyte in early preimplantation mouse embryos. *Biol Reprod*. 1998;59:225–32.
157. Lane M. Mechanisms for managing cellular and homeostatic stress in vitro. *Theriogenology*. 2001;55:225–36.
158. Chi HJ, Park JS, Yoo CS, Kwak SJ, Son HJ, Kim SG, et al. Effect of evaporation-induced osmotic changes in culture media in a dry-type incubator on clinical outcomes in in vitro fertilization-embryo transfer cycles. *Clin Exp Reprod Med*. 2020;47: 284–92.
159. Mestres E, Garcia-Jimenez M, Casals A, Cohen J, Acacio M, Villamar A, et al. Factors of the human embryo culture system that may affect media evaporation and osmolality. *Hum Reprod*. 2021;36:605–13.
160. Leese HJ. The formation and function of oviduct fluid. *J Reprod Fertil*. 1988;82:843–56.
161. Bolton VN, Cutting R, Clarke H, Brison DR. ACE consensus meeting report: Culture systems. *Hum Fertil (Camb)*. 2014;17: 239–51.
162. Wiley LM, Yamami S, Van Muyden D. Effect of potassium concentration, type of protein supplement, and embryo density on mouse preimplantation development in vitro. *Fertil Steril*. 1986;45: 111–9.
163. Paria BC, Dey SK. Preimplantation embryo development in vitro: Cooperative interactions among embryos and role of growth factors. *Proc Natl Acad Sci U S A*. 1990;87:4756–60.
164. Lane M, Gardner DK. Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. *Hum Reprod*. 1992;7:558–62.
165. Salahuddin S, Ookutsu S, Goto K, Nakanishi Y, Nagata Y. Effects of embryo density and co-culture of unfertilized oocytes on embryonic development of in-vitro fertilized mouse embryos. *Hum Reprod*. 1995;10:2382–5.
166. Gardner DK, Lane MW, Lane M. Development of the inner cell mass in mouse blastocyst is stimulated by reducing the embryo: Incubation volume ratio. *Hum Reprod* 1997;12 P–132.
167. Ahern TJ, Gardner DK. Culturing bovine embryos in groups stimulates blastocyst development and cell allocation to the inner cell mass. *Theriogenology*. 1998;49:194.
168. Ebner T, Shebl O, Moser M, Mayer RB, Arzt W, Tews G. Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and live birth. *Reprod Biomed Online*. 2010;21:762–8.
169. Rijnders PM, Jansen CA. Influence of group culture and culture volume on the formation of human blastocysts: A prospective randomized study. *Hum Reprod*. 1999;14:2333–7.
170. Gardner DK. Improving embryo culture and enhancing pregnancy rate. In: Female Infertility Therapy: Current Practise. Shoham Z, Howles C, Jacobs H (eds). London: Martin Dunitz, pp. 283–99, 1998.
171. Kelley RL, Gardner DK. Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. *Reprod Biomed Online*. 2016;33(5):537–49.
172. Truong TT, Soh YM, Gardner DK. Antioxidants improve mouse preimplantation embryo development and viability. *Hum Reprod*. 2016;31:1445–54.
173. Kelley RL, Gardner DK. In vitro culture of individual mouse preimplantation embryos: The role of embryo density, microwells, oxygen, timing and conditioned media. *Reprod Biomed Online*. 2017;34:441–54.
174. Vajta G, Parmegiani L, Machaty Z, Chen WB, Yakovenko S. Back to the future: Optimised microwell culture of individual human preimplantation stage embryos. *J Assist Reprod Genet*. 2021;38:2563–74.
175. Fancsovits P, Pribenszky C, Lehner A, Murber A, Kaszas Z, Nemes A, et al. Prospective-randomized study comparing clinical outcomes of IVF treatments where embryos were cultured individually or in a microwell group culture dish. *Biol Futur*. 2022;73: 229–36.
176. Wilson M, Hartke K, Kiehl M, Rodgers J, Brabec C, Lyles R. Integration of blastocyst transfer for all patients. *Fertil Steril*. 2002;77:693–6.
177. Marek D, Langley M, Gardner DK, Confer N, Doody KM, Doody KJ. Introduction of blastocyst culture and transfer for all patients in an in vitro fertilization program. *Fertil Steril*. 1999;72:1035–40.
178. Menezo YJ, Guerin JF, Czyba JC. Improvement of human early embryo development in vitro by coculture on monolayers of Vero cells. *Biol Reprod*. 1990;42:301–6.
179. Lopata A. The neglected human blastocyst. *J Assist Reprod Genet*. 1992;9:508–12.
180. Olivennes F, Hazout A, Lelaidier C, Freitas S, Fanchin R, de Ziegler D, et al. Four indications for embryo transfer at the blastocyst stage. *Hum Reprod*. 1994;9:2367–73.
181. Scholtes MC, Zeilmaker GH. A prospective, randomized study of embryo transfer results after 3 or 5 days of embryo culture in in vitro fertilization. *Fertil Steril*. 1996;65:1245–8.
182. Barnes FL. The effects of the early uterine environment on the subsequent development of embryo and fetus. *Theriogenology*. 2000;53:649–58.
183. Walker KJ, Green MP, Gardner DK. Spatial asynchronous transfer of cleavage-stage mouse embryos to the uterus compromises fetal development. *Mol Reprod Dev*. 2015;82:80.
184. Ertzeid G, Storeng R. Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice. *J Reprod Fertil*. 1992;96:649–55.
185. Ertzeid G, Storeng R, Lyberg T. Treatment with gonadotropins impaired implantation and fetal development in mice. *J Assist Reprod Genet*. 1993;10:286–91.
186. Van der Auwera I, Pijnenborg R, Koninckx PR. The influence of in-vitro culture versus stimulated and untreated oviductal environment on mouse embryo development and implantation. *Hum Reprod*. 1999;14:2570–4.
187. Taylor DM, Ray PF, Ao A, Winston RM, Handyside AH. Paternal transcripts for glucose-6-phosphate dehydrogenase and adenosine deaminase are first detectable in the human preimplantation embryo at the three- to four-cell stage. *Mol Reprod Dev*. 1997;48:442–8.

188. Tesarik J. Developmental failure during the preimplantation period of human embryogenesis. In: *The Biological Basis of Early Human Reproductive Failure*. Van Blerkom J (ed). New York, NY: Oxford University Press, pp. 327–44, 1994.
189. Sandalinas M, Sadowy S, Alikani M, Calderon G, Cohen J, Munne S. Developmental ability of chromosomally abnormal human embryos to develop to the blastocyst stage. *Hum Reprod*. 2001;16:1954–8.
190. Vega M, Breborowicz A, Moshier EL, McGovern PG, Keltz MD. Blastulation rates decline in a linear fashion from euploid to aneuploid embryos with single versus multiple chromosomal errors. *Fertil Steril*. 2014;102:394–8.
191. Adler A, Lee HL, McCulloch DH, Ampelouquio E, Clarke-Williams M, Wertz BH, et al. Blastocyst culture selects for euploid embryos: Comparison of blastomere and trophectoderm biopsies. *Reprod Biomed Online*. 2014;28:485–91.
192. Fanchin R, Righini C, Olivennes F, Taylor S, de Ziegler D, Frydman R. Uterine contractions at the time of embryo transfer alter pregnancy rates after in-vitro fertilization. *Hum Reprod*. 1998;13:1968–74.
193. Lesny P, Killick SR, Tetlow RL, Robinson J, Maguiness SD. Uterine junctional zone contractions during assisted reproduction cycles. *Hum Reprod Update*. 1998;4:440–5.
194. Fanchin R, Ayoubi JM, Righini C, Olivennes F, Schonauer LM, Frydman R. Uterine contractility decreases at the time of blastocyst transfers. *Hum Reprod*. 2001;16:1115–9.
195. Fragouli E, Lenzi M, Ross R, Katz-Jaffe M, Schoolcraft WB, Wells D. Comprehensive molecular cytogenetic analysis of the human blastocyst stage. *Hum Reprod*. 2008;23:2596–608.
196. Schoolcraft WB, Fragouli E, Stevens J, Munne S, Katz-Jaffe MG, Wells D. Clinical application of comprehensive chromosomal screening at the blastocyst stage. *Fertil Steril*. 2010;94: 1700–6.
197. Mastenbroek S, Twisk M, van Echten-Arends J, Sikkema-Raddatz B, Korevaar JC, Verhoeve HR, et al. In vitro fertilization with preimplantation genetic screening. *N Engl J Med*. 2007;357: 9–17.
198. Scott RT Jr., Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: A randomized and paired clinical trial. *Fertil Steril*. 2013;100:624–30.
199. Papanikolaou EG, Camus M, Kolibianakis EM, Van Landuyt L, Van Steirteghem A, Devroey P. In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. *N Engl J Med*. 2006;354:1139–46.
200. Papanikolaou V. Early pregnancy loss is significantly higher after day 3 single embryo transfer than after day 5 single blastocyst transfer in GnRH antagonist stimulated IVF cycles. *RBM Online*. 2006;12:60–5.
201. Clua E, Rodriguez I, Arroyo G, Racca A, Martinez F, Polyzos NP. Blastocyst versus cleavage embryo transfer improves cumulative live birth rates, time and cost in oocyte recipients: A randomized controlled trial. *Reprod Biomed Online*. 2022;44: 995–1004.
202. Hunault CC, Eijkemans MJ, Pieters MH, te Velde ER, Habbema JD, Fauser BC, et al. A prediction model for selecting patients undergoing in vitro fertilization for elective single embryo transfer. *Fertil Steril*. 2002;77:725–32.
203. Balaban B, Urman B, Alatas C, Mercan R, Aksoy S, Isiklar A. Blastocyst-stage transfer of poor-quality cleavage-stage embryos results in higher implantation rates. *Fertil Steril*. 2001;75: 514–8.
204. Schoolcraft WB, Gardner DK. Blastocyst culture and transfer increases the efficiency of oocyte donation. *Fertil Steril*. 2000;74:482–6.
205. Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: A prospective randomized trial. *Fertil Steril*. 2004;81:551–5.
206. Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF. A formula for scoring human embryo growth rates in in vitro fertilization: Its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *J In Vitro Fert Embryo Transf*. 1986;3:284–95.
207. Steer CV, Mills CL, Tan SL, Campbell S, Edwards RG. The cumulative embryo score: A predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. *Hum Reprod*. 1992;7: 117–9.
208. Scott L. The biological basis of non-invasive strategies for selection of human oocytes and embryos. *Hum Reprod Update*. 2003;9:237–49.
209. Li Z, Wang YA, Ledger W, Edgar DH, Sullivan EA. Clinical outcomes following cryopreservation of blastocysts by vitrification or slow freezing: A population-based cohort study. *Hum Reprod*. 2014;29:2794–801.
210. Takahashi K, Mukaida T, Goto T, Oka C. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: A 4-year follow-up study. *Fertil Steril*. 2005;84:88–92.
211. Roy TK, Bradley CK, Bowman MC, McArthur SJ. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. *Fertil Steril*. 2014;101:1294–301.
212. Xie Y, Wang F, Puscheck EE, Rappolee DA. Pipetting causes shear stress and elevation of phosphorylated stress-activated protein kinase/jun kinase in preimplantation embryos. *Mol Reprod Dev*. 2007;74:1287–94.
213. Gardner DK, Reed L, Linck D, Sheehan C, Lane M. Quality control in human in vitro fertilization. *Semin Reprod Med*. 2005;23:319–24.
214. Khan Z, Wolff HS, Fredrickson JR, Walker DL, Daftary GS, Morbeck DE. Mouse strain and quality control testing: Improved sensitivity of the mouse embryo assay with embryos from outbred mice. *Fertil Steril*. 2013;99:847–54 e2.
215. Silverman IH, Cook CL, Sanfilippo JS, Yussman MA, Schultz GS, Hilton FH. Ham's F-10 constituted with tap water supports mouse conceptus development in vitro. *J In Vitro Fert Embryo Transf*. 1987;4:185–7.
216. George MA, Braude PR, Johnson MH, Sweetnam DG. Quality control in the IVF laboratory: in-vitro and in-vivo development of mouse embryos is unaffected by the quality of water used in culture media. *Hum Reprod*. 1989;4:826–31.
217. Fissore RA, Jackson KV, Kiessling AA. Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid. *Biol Reprod*. 1989;41:835–41.
218. Flood LP, Shirley B. Reduction of embryotoxicity by protein in embryo culture media. *Mol Reprod Dev*. 1991;30:226–31.
219. Wolff HS, Fredrickson JR, Walker DL, Morbeck DE. Advances in quality control: Mouse embryo morphokinetics are sensitive markers of in vitro stress. *Hum Reprod*. 2013;28: 1776–82.
220. Gardner DK, Truong TT. Antioxidants and antifreeze proteins in cryopreservation and vitrification. In: *Cryopreservation in Assisted Reproduction: A Practitioner's Guide to Methods, Management and Organisation*. Nagy ZP, Varghese A, Agarwal A (eds). New York, NY: Springer, 2024.
221. Truong T, Gardner DK. Antioxidants improve IVF outcome and subsequent embryo development in the mouse. *Hum Reprod*. 2017;32:2404–13.
222. Truong TT, Gardner DK. Antioxidants increase blastocyst cryo-survival and viability post-vitrification. *Hum Reprod*. 2020;35: 12–23.
223. Gardner DK, Kuramoto T, Tanaka M, Mitzumoto S, Montag M, Yoshida A. Prospective randomized multicentre comparison on sibling oocytes comparing g-series media system with antioxidants versus standard G-Series media system. *Reprod Biomed Online*. 2020;40:637–44.

224. Hannan NJ, Paiva P, Meehan KL, Rombauts LJ, Gardner DK, Salamonsen LA. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. *Endocrinology*. 2011;152:4948–56.
225. Thouas GA, Dominguez F, Green MP, Vilella F, Simon C, Gardner DK. Soluble ligands and their receptors in human embryo development and implantation. *Endocr Rev*. 2015;36:92–130.
226. Binder NK, Evans J, Gardner DK, Salamonsen LA, Hannan NJ. Endometrial signals improve embryo outcome: Functional role of vascular endothelial growth factor isoforms on embryo development and implantation in mice. *Hum Reprod*. 2014;29:2278–86.
227. Ziebe S, Loft A, Povlsen BB, Erb K, Agerholm I, Aasted M, et al. A randomized clinical trial to evaluate the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization. *Fertil Steril*. 2013;99:1600–9.
228. Karagenc L, Lane M, Gardner DK. Granulocyte-macrophage colony-stimulating factor stimulates mouse blastocyst inner cell mass development only when media lack human serum albumin. *Reprod Biomed Online*. 2005;10:511–8.
229. Kawamura K, Chen Y, Shu Y, Cheng Y, Qiao J, Behr B, et al. Promotion of human early embryonic development and blastocyst outgrowth in vitro using autocrine/paracrine growth factors. *PLoS One*. 2012;7:e49328.
230. Gurner KH, Truong TT, Harvey AJ, Gardner DK. A combination of growth factors and cytokines alter preimplantation mouse embryo development, foetal development and gene expression profiles. *Mol Hum Reprod*. 2020;26:953–70.
231. Suh RS, Phadke N, Ohl DA, Takayama S, Smith GD. Rethinking gamete/embryo isolation and culture with microfluidics. *Hum Reprod Update*. 2003;9:451–61.
232. Wheeler MB, Walters EM, Beebe DJ. Toward culture of single gametes: The development of microfluidic platforms for assisted reproduction. *Theriogenology*. 2007;68(Suppl 1):S178–89.
233. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod*. 1996;11:1975–8.
234. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, et al. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod*. 2004;19:2319–24.
235. McLennan HJ, Blanch AJ, Wallace SJ, Ritter LJ, Heinrich SL, Gardner DK, et al. Nano-liter perfusion microfluidic device made entirely by two-photon polymerization for dynamic cell culture with easy cell recovery. *Sci Rep*. 2023;13:562.
236. Gardner DK. The way to improve ART outcomes is to introduce more technologies in the laboratory. *Reprod Biomed Online*. 2022;44:389–92.
237. Vajta G, Parmegiani L. Cleavage stage vs. blastocyst transfer: A more considerate analysis suggested. *Hum Reprod*. 2020;35:2399–400.
238. Crosby IM, Gandolfi F, Moor RM. Control of protein synthesis during early cleavage of sheep embryos. *J Reprod Fertil*. 1988;82:769–75.
239. Rieger D, Loskutoff NM, Betteridge KJ. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. *Reprod Fertil Dev*. 1992;4:547–57.
240. Liu Z, Foote RH. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O₂. *Biol Reprod*. 1995;53:786–90.
241. Lindenbaum A. A survey of naturally occurring chelating ligands. *Adv Exp Med Biol*. 1973;40:67–77.
242. Wu G, Morris SM Jr. Arginine metabolism: Nitric oxide and beyond. *Biochem J*. 1998;336(Pt 1):1–17.
243. Martin PM, Sutherland AE, Van Winkle LJ. Amino acid transport regulates blastocyst implantation. *Biol Reprod*. 2003;69:1101–8.
244. Martin PM, Sutherland AE. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. *Dev Biol*. 2001;240:182–93.
245. Hardy K, Robinson FM, Paraschos T, Wicks R, Franks S, Winston RM. Normal development and metabolic activity of preimplantation embryos in vitro from patients with polycystic ovaries. *Hum Reprod*. 1995;10:2125–35.
246. Simon C, Garcia Velasco JJ, Valbuena D, Peinado JA, Moreno C, Remohi J, et al. Increasing uterine receptivity by decreasing estradiol levels during the preimplantation period in high responders with the use of a follicle-stimulating hormone step-down regimen. *Fertil Steril*. 1998;70:234–9.
247. Ertzeid G, Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. *Hum Reprod*. 2001;16:221–5.
248. Kelley RL, Kind KL, Lane M, Robker RL, Thompson JG, Edwards LJ. Recombinant human follicle-stimulating hormone alters maternal ovarian hormone concentrations and the uterus and perturbs fetal development in mice. *Am J Physiol Endocrinol Metab*. 2006;291:E761–70.
249. Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development*. 2000;127:4195–202.
250. Gardner DK, Lane KS. High protein diet inhibits inner cell mass formation and increases apoptosis in mouse blastocysts developed in vivo by increasing the levels of ammonium in the reproductive tract. *Reprod, Fertil Dev*. 2004;16:190.

16

EMBRYO SELECTION THROUGH THE ANALYSIS OF MORPHOLOGY AND PHYSIOLOGY

Denny Sakkas and David K. Gardner

Introduction

Worldwide the utilization of assisted reproductive technologies (ART) continues to increase annually. In 2006, more than one million cycles were registered in the international report on ART monitoring [1]. Current data indicate that more than two and a half million ART cycles are being performed each year, with a total of over eight million babies born worldwide since the birth of Louise Brown [2–4]. Well, over a million treatment cycles are initiated annually in the United States, Europe, Australia, and New Zealand alone [1–3, 5]. This increasing trend of ART utilization has been driven by the steady improvement in delivery rates, improved access to care in many areas, and the relative ineffectiveness of other treatment options. The proportion of infants after ART in Europe, the United States, and Australia/New Zealand [6] now ranges from 1.9% to 4.1% of all children born [3, 7].

Historically acceptable success rates through in vitro fertilization (IVF) were attained, in many cases, only through the simultaneous transfer of multiple embryos. However, this trend has changed dramatically. In the United States, an average of 2.8 embryos were transferred in women <38 years of age in 2003 compared to 1.2 embryos per patient in 2019 [8]. A further trend has been a shift to transferring embryos after cryo-storage into a more receptive uterine environment, which has also led to lower numbers of embryos transferred [8].

The risks to both mother and baby related to multiple gestations are well documented and include maternal hypertension, preterm delivery, low birth weight, and a dramatic increase in the relative risk for cerebral palsy (reviewed by [9–12]). These complications lead to a higher incidence of medical, perinatal, and neonatal complications and a tenfold increase in healthcare costs compared to a singleton delivery [13]. Decreasing the prevalence of multiple gestations in IVF can only be achieved by the transfer of a single embryo.

In many countries, including Norway, Sweden, Denmark, Belgium, England, Italy and Germany, legal restrictions/medical guidelines have been implemented for several years that govern the number of embryos that can be transferred in a given IVF cycle. For example, in most Scandinavian countries and Belgium, the government has set a legal limit of single embryo transfer (i.e. only one embryo to be transferred per cycle) for specific patient groups, while many other European countries have restricted the number of transferred embryos to a maximum of two. In other parts of the world, where no legal restrictions exist, the onus is on the individual clinic (along with the patient) to decrease the number of embryos transferred so that an acceptable balance can be achieved between the risks associated with multiple gestations and “acceptable” pregnancy rates. In Australia and New Zealand, this was achieved by clinicians and patients willingly shifting to single embryo transfer, with the proportion increasing

from 69.7% in 2009 to 79.2% in 2013 to 93% in 2020 [6]. Of note, in the USA, the Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology have now stipulated under what circumstances single embryo transfer should take place [14]. In China, the number of embryos transferred has also shifted to just under an average of two per transfer [15]. Current indications are that in the future all countries currently lacking legislation will be compelled via legal, financial, and/or moral obligation to restrict the number of embryos transferred in order to minimize the risk of multiple gestations.

A major issue in limiting the number of embryos transferred remains the apparent inability to accurately estimate the reproductive potential of individual embryos within a cohort of embryos using the existing selection techniques, which largely depends upon morphological evaluation. Faced with the scenario that we, the worldwide IVF community, will in the future have to select only one or, at most, two embryos for transfer, will force us to make certain choices. There has also been a debate on the value of milder stimulation protocols that generate a lower number of eggs at collection. The generation of a smaller number of oocytes has been argued to lead to a greater percentage of viable embryos within a given cohort [16, 17]. Contrary to this argument, a higher egg yield has been shown to improve cumulative live birth rates [18], and can provide two or more live births with just one stimulation cycle in almost a quarter of patients [19–24]. With the implementation of vitrification, the fear of loss of embryo quality after cryopreserving has also been largely removed. The scenario of creating more embryos and performing a frozen embryo transfer will place a greater onus on improving the selection process for defining the quality of individual embryos so that the ones we choose for transfer are more likely to implant, thereby significantly decreasing the time to pregnancy. This chapter will discuss several strategies in selection criteria that will help us achieve this second choice.

Morphology as an assessment tool

For more than 30 years, morphological assessment has been the primary means for embryologists to select which embryo(s) to replace. From the early years of IVF, it was noted that embryos that cleave faster, and those of better morphological appearance, were more likely to lead to a pregnancy [25, 26]. Morphological assessment systems subsequently evolved over the past three decades, and in addition to the classical parameters of cell number and fragmentation, numerous other characteristics have been examined, including pronucleate oocyte morphology, early cleavage to the 2-cell stage, top-quality embryos on successive days, and various forms of sequential assessment of embryos (see reviews [27, 28]). In addition, the ability to culture and assess blastocyst stage embryos has also significantly improved embryo

selection on the basis of morphology [29]. Although morphology has proved a difficult target to marry with viability, it does provide us a gross overall vision on key milestones an embryo should obtain at specific times [30]. This has been better appreciated in the past few years, when we have also seen the advent of commercially available video-imaging technologies which bring new light to how we interpret and use the morphological features of the embryo (see Chapter 17 and [31]). Here we briefly describe some of the historical papers that examined key morphogenetic events and the key times at which they should take place in the laboratory.

The pronucleate oocyte

The many transformations that take place during the fertilization process make this a highly dynamic stage to assess. The oocyte contains the majority of the developmental materials, maternal mRNA, for ensuring that the embryo reaches the 4- to 8-cell stage. In human embryos, embryonic genome activation has been shown to occur between the 4- and 8-cell stages [32]. The quality of the oocyte, therefore, plays the lead role in determining early embryo development and subsequent viability.

A number of studies postulated that embryo quality can be predicted at the pronucleate oocyte stage. Separate studies by Tesarik and Scott [33, 34] concentrated on the predictive value of the nucleoli. Tesarik and Greco [34] proposed that the normal and abnormal morphology of the pronuclei were related to the developmental fate of human embryos. They retrospectively assessed the number and distribution of nucleolar precursor bodies (NPB) in each pronucleus of fertilized oocytes that led to embryos that implanted. The characteristics of the zygotes were then compared to those that led to failures in implantation. The features that were shared by zygotes that had 100% implantation success were (i) the number of NPB in both pronuclei never differed by more than three, and (ii) the NPB were always polarized or non-polarized in both pronuclei but never polarized in one pronucleus and not in the other. Pronucleate oocytes not showing this criteria were more likely to develop into pre-implantation embryos that had poor morphology and/or experienced cleavage arrest. The presence of at least one embryo, which had shown the preceding criteria at the pronuclear stage in those transferred, led to a pregnancy rate of 22/44 (50%) compared to only 2/23 (9%) when none were present.

A further criterion of pronucleate oocytes that may affect embryo morphology is the orientation of pronuclei relative to the polar bodies. Oocyte polarity is clearly evident in non-mammalian species. In mammals, the animal pole of the oocyte may be estimated by the location of the first polar body, whereas after fertilization, the second polar body marks the embryonic pole [35]. In human oocytes, a differential distribution of various factors within the oocyte has been described and anomalies in the distribution of these factors, in particular the side of the oocyte believed to contain the animal pole, are thought to affect embryo development and possibly fetal growth [36, 37]. Following from this hypothesis, Garello et al. [38] examined pronuclear orientation, polar body placement, and embryo quality to ascertain if a link existed between a plausible polarity of oocytes at the pronuclear stage and further development. The most interesting observation involved the calculation of angle β (Figure 16.1), which represented the angle between a line drawn through the axis of the pronuclei and the position of the furthest polar body. It was determined that as the angle β increased there was a concurrent

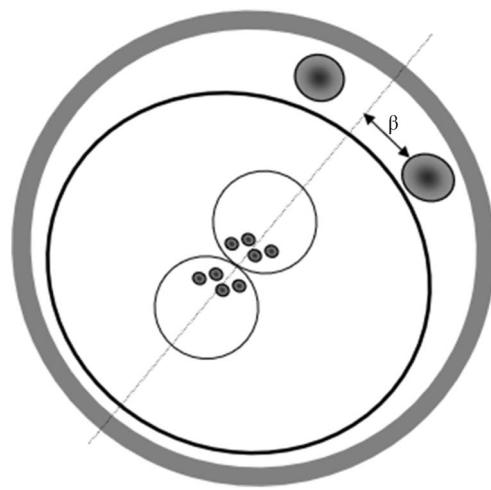


FIGURE 16.1 Ideal features shared by pronuclear embryos that have high viability as described by Tesarik and Greco [32], Garello et al. [37], and Scott and Smith [39]: (i) the number of nucleolar precursor bodies (NPB) in both pronuclei never differed by more than 3; (ii) the NPB are always polarized or non-polarized in both pronuclei but never polarized in one pronucleus and not in the other; (iii) the angle from the axis of the pronuclei and the furthest polar body is less than 50°.

decrease in the morphological quality of pre-implantation stage human embryos. Hence, it was postulated that the misalignment of the polar body might be linked to cytoplasmic turbulence disturbing the delicate polarity of the zygote. To this day, the question of polarity in the oocyte and its importance in influencing embryo viability is still not well understood [39].

In a further study, Scott and Smith [40] devised an embryo score on day 1 on the basis of alignment of pronuclei and nucleoli, the appearance of the cytoplasm, nuclear membrane breakdown, and cleavage to the 2-cell stage. Patients who had an overall high embryo score (≥ 15) had a pregnancy and implantation rate of 34/48 (71%) and 49/175 (28%), respectively, compared to only 4/49 (8%) and 4/178 (2%) in the low embryo score group. The use of pronuclear scoring was extensively reviewed by Scott [41]. The timing of pronuclear events has been confirmed to be correlative to implantation potential by video imaging. Aguilar et al. [42] showed that the timings at which second polar body extrusion (3.3–10.6 hours), pronuclear fading (22.2–25.9 hours), and length of S-phase (5.7–13.8 hours) occurred were all linked successfully to embryo implantation. The same group also confirmed that the method of fertilization, intracytoplasmic sperm injection (ICSI) or routine IVF, was also important in determining how these parameters should be evaluated [43]. Interestingly, these features may still provide some insights into viability assessment even with the advent of morphokinetics [44]. A recent study did however strengthen the concept that the closer an embryo is observed to the time of implantation the more relevant the information in relation to viability. Ezoe et al. [44] found that non-juxtaposition and asynchronous pronuclear breakdown was associated with abnormal mitosis at the first cleavage and impaired pre-implantation development. However, embryos displaying abnormal pronuclear breakdown also formed blastocysts which led to live births. They suggested blastocyst transfer as a more appropriate culture strategy.

Cleavage stage embryos

Although the use of blastocyst stage culture has been more widely accepted, selection at the cleavage stage for transfer, based on cell number and morphology [25] is still prevalent. In some cases, this is still driven by legal restrictions in certain countries. For example, the German embryo protection law, passed in 1991, stipulates that no more than three embryos can be created per cycle of IVF and all three, regardless of their quality, must be transferred. In relation to assessment of cleavage-stage embryos, some of the key studies were originally presented by Gerris et al. [45] and Van Royen et al. [46], who employed strict embryo criteria to select single embryos for transfer. These did not however differ greatly to papers published in the 1980s by Cummins et al. [25] who also described key cleavage events linked with viability. What constitutes a “top” quality embryo? These “top” quality embryos had the following characteristics: four or five blastomeres on day 2 and at least seven blastomeres on day 3 after fertilization, absence of multi-nucleated blastomeres, and <20% of fragments on day 2 and day 3 after fertilization. When these criteria were utilized in a prospective randomized clinical trial comparing single and double embryo transfers, it was found that in 26 single embryo transfers where a top-quality embryo was available an implantation rate of 42.3% and ongoing pregnancy rate of 38.5% was obtained. In 27 double embryo transfers, an implantation rate of 48.1% and ongoing pregnancy rate of 74% was obtained. A larger study analysing the outcome of 370 consecutive single top-quality embryo transfers in patients younger than 38 years for pregnancy showed that the pregnancy rate after single top-quality embryo transfer was 51.9% [47].

The majority of studies that have used and report embryo selection criteria on the basis of cell number and morphology do so by stating that embryos were selected on day 2 or day 3. As discussed by Bavister [48], one of the most critical factors in determining selection criteria was to ascertain strict time points to compare the embryos. Sakkas and colleagues therefore used cleavage to the 2-cell stage at 25 hours post insemination or microinjection as the critical time point for selecting embryos [49–51]. In a larger series of patients, it was found that 45% of patients undergoing IVF or ICSI have early cleaving 2-cell embryos. Patients who have early cleaving 2-cell stage embryos allocated for transfer on day 2 or 3 have significantly higher implantation and pregnancy rates [49]. Furthermore, nearly 50% of the patients who have two early cleaving 2-cell embryos transferred achieve a clinical pregnancy (Figure 16.2). The most convincing data supporting the usefulness of early cleaving 2-cell embryos is that provided by single embryo transfer [52, 53]. In one study, Salumets et al. [52] showed that when transferring single embryos a significantly higher clinical pregnancy rate was observed after transfer of early cleaving (50%) rather than non-early cleaving (26.4%) embryos. The embryos that cleave early to the 2-cell stage have also been reported to have a significantly higher blastocyst formation rate [54, 55]. Another study by Guerif et al. [54] reported the sequential growth of 4042 embryos individually cultured from day 1 to day 5 or 6. Interestingly, early cleavage and cell number on day 2 were the most powerful parameters to predict the development of a good morphology blastocyst at day 5. Video imaging has aided in refining these timing events and now numerous algorithms exist [56, 57] which help incorporate both cleavage and timing in predicting both blastocyst development and implantation potential [58, 59].

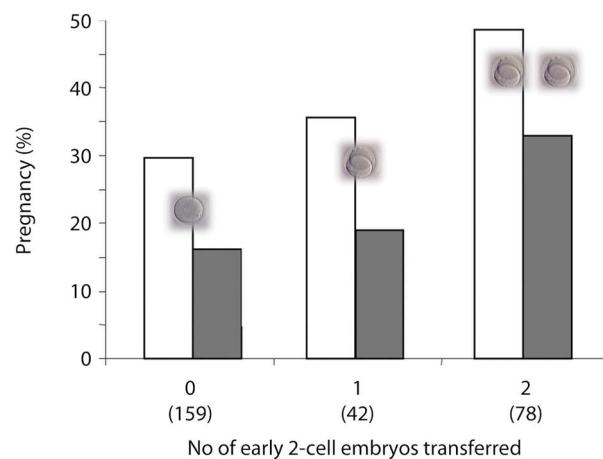


FIGURE 16.2 The percentage of clinical pregnancies (light columns) and implantation rate (dark columns) in relation to whether patients had zero, one, or two early cleavage embryos transferred. The numbers in parentheses indicate the number of cycles per group.

Morulae stage embryos

One somewhat overlooked stage has been the morula stage. Why this has not been used as an assessment tool is interesting but mostly stems from a need to not over-observe embryos and lack of definitive historical morphological assessment during this stage. With the introduction of time-lapse analysis, we can now readily visualize and analyse key morphogenetic events around the time at which the first epithelium of the conceptus is formed. Studies now indicate that a day 4 scoring system could be successfully adopted and implemented [60] and may provide SET rates similar to day 5 SETs [61]. The adoption of such a strategy has however still not been thoroughly evaluated.

Development to the blastocyst stage

Blastocyst stage transfer has become standard clinical care in several countries, due to a number of factors. These include: the commercial availability of sequential, one-step, and time-lapse culture media; improvements in blastocyst cryopreservation made possible through vitrification; [62–64] and finally the move to the biopsy of the trophectoderm for PGT-A [31, 65–68].

The quality of blastocyst obtained is however of critical importance. As with the scoring of embryos during the cleavage stages, timing and morphology are key in selecting the best blastocyst. The scoring assessment for blastocysts devised by Gardner and Schoolcraft [69] is one of the most widely adopted. In effect, even the Alpha Scoring System is a numerical interpretation of the Gardner scale [70]. The Gardner scoring system is based on the expansion state of the blastocyst and on the consistency of the inner cell mass (ICM) and trophectoderm cells (Figure 16.3). Examples of high-quality blastocysts are shown in Figure 16.4. Using such a grading system it was determined that when two high-scoring blastocysts (>3AA), i.e. expanded blastocoel with compacted ICM and cohesive trophectoderm epithelium are transferred, a clinical pregnancy and implantation rate of >80% and 69% can be attained [71]. When two blastocysts not achieving these scores (<3AA) are transferred, the clinical pregnancy and implantation rate are significantly lower, 50% and 33%, respectively [72].

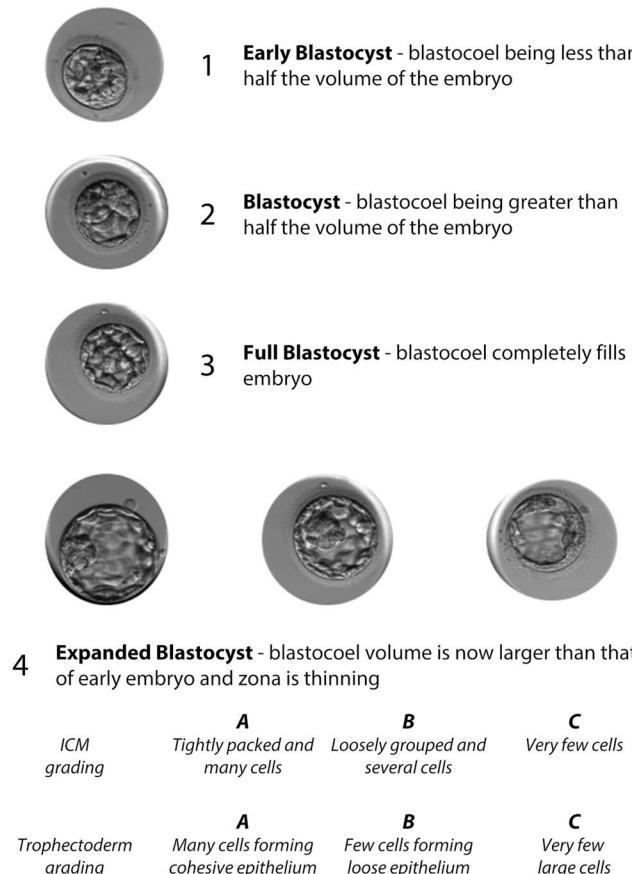


FIGURE 16.3 The blastocyst grading system. (Modified from Gardner and Schoolcraft [69].)

Although reduced from the values obtained with top-scoring blastocysts, it is evident that early blastocysts on day 5 still have high developmental potential.

More recent detailed analyses of whether the ICM or trophectoderm provides greater predictive weight for embryo selection concluded that the predictive strength of the trophectoderm grade was greater compared to the ICM for selecting the best blastocyst for embryo replacement [73]. It has been suggested that even though ICM is important, a strong trophectoderm layer is

essential at this stage of embryo development, allowing successful hatching and implantation. This has subsequently been validated by a number of studies [74–78] which all highlighted the need for a strong trophectoderm grading in relation to pregnancy. Interestingly, one study found that a poor ICM grading was also related to higher miscarriage rates [79].

Van den Abbeel and colleagues [79] have also provided data on the importance of the ICM grade when assessing the blastocyst, reporting that all three parameters of the blastocyst (degree of expansion, ICM, and TE quality) were significantly associated with pregnancy and live birth rates. It was further determined that transfer of blastocysts with an “A” grade ICM reduces the incidence of pregnancy loss [79], and that ICM grade is positively associated with birth weight [80]. Physiologically, the TE and ICM do not exist in isolation, but rather are a functional unit. Although the TE creates a unique environment for the ICM by the synthesis of blastocoel fluid, it is the ICM itself that regulates the proliferation and activity of the trophectoderm in the mouse blastocyst [81–83]. Hence, it appears prudent to utilize both the ICM and TE grades in decisions regarding the fate of an embryo. Interestingly, it was also recently shown that a blastocyst with a low overall grading was associated with a higher chance of female baby (48% vs 42%, adjusted OR = 1.26 [1.13, 1.39]) and a higher rate of caesarean section (C-section; 71% vs 68%, adjusted OR = 1.15 [1.02, 1.29]) [84]. Finally, it is also evident that even poor-grade blastocysts (CC) have potential, although greatly reduced when compared to high-quality blastocysts [85].

In addition to the ICM and TE grade the timing of blastocyst formation is also crucial, in particular when performing fresh transfers [86]. The timing of blastocyst formation however is less important when considering frozen transfers. In theory, achieving the blastocyst stage at day 5 suggests that they may be of higher quality. Recent retrospective cohort studies using vitrification have demonstrated that the live birth rate may be slightly higher with day 5 versus day 6 vitrified-warmed blastocysts [73, 87, 88]. It is also clear now that even blastocysts forming on day 7 have respectable live birth rates and that when frozen and transferred back to a re-synchronized uterus they can add significantly to a patient’s chance of success [89, 90].

Some groups have also attempted to correlate blastocyst rates and quality with overall ploidy status of the embryo. Interestingly, identifying euploid embryos by PGT-A appeared to override blastocyst morphological grade and day of cryopreservation, as regardless of grade their live birth rates were not significantly



FIGURE 16.4 Day 5 human blastocysts. Using the grading system reported by Gardner and Schoolcraft [69]. Blastocysts in (a) and (b) would both score 4AA; the embryo in (c) would only score 4CA due to the apparent absence of an ICM, in spite of the development of an excellent trophectoderm.

different [88]. Predicting the euploid blastocyst by morphology and time-lapse has become a particular goal but has not been conclusive. In one study, Campbell and colleagues reported that the timing of formation of the blastocoel was delayed in aneuploid embryos [91]. Time to the start of blastulation of <100 hours after insemination and the morphokinetic scoring system used in the time-lapse group were independently associated with implantation. The association between cleavage parameters and prediction of aneuploidy warrants further study [92]. Recently, the use of artificial intelligence to examine static images of blastocysts has shown promise [93]. In this study a total of 1231 embryo images were classed as good prognosis if euploid and implanted or poor prognosis if aneuploid and failed to implant. An accuracy of 0.70 was obtained using an embryo ranking artificial intelligence (AI) algorithm, with positive predictive value of 0.79 for predicting euploidy. The use of AI, in reference to embryo selection, is providing some exciting promise with high predictability [94] ([Chapters 18 and 19](#)).

A strategy for selecting the best embryo by morphology

The preceding selection criteria have all shown that they generate some benefit in identifying individual embryos that have a high viability. Curiously, one thing that video imaging seems to be teaching us is to not only investigate the things that go right but also the things that go wrong. Although video imaging has aimed to develop selection algorithms looking for positive selection features related to embryo implantation potential, it has also shown us that numerous events can be used to deselect embryos from the transfer pool. One of the most evident deselection events seems to be direct cleavage to the 3-cell stage [95].

How do we implement a strategy for selecting a single embryo when we have many embryos to choose from? A few schools of thought are now being adopted for embryo selection. Previously, it was suggested that a multiple-step scoring system that encompasses all the preceding criteria would be the best approach. The use of sequential scoring systems has been shown to be beneficial by a number of authors [46, 55, 96]. The advent of machine learning with time-lapse has superseded this manual process, as all

the preceding criteria can be better integrated in an automated fashion.

18–19 hours post insemination/ICSI ([Figure 16.1](#)): Identification of two pronuclei embryos. The pronuclei are examined for:

- a. symmetry
- b. the presence of even numbers of NPB
- c. the positioning of the polar bodies

25–26 hours post insemination/ICSI ([Figure 16.5](#)):

- a. embryos that have already cleaved to the 2-cell stage
- b. zygotes that have progressed to nuclear membrane breakdown

42–44 hours post insemination/ICSI ([Figure 16.5](#)):

- a. number of blastomeres should be greater or equal to four
- b. fragmentation of less than 20%
- c. no multi-nucleated blastomeres

66–68 hours post insemination/ICSI ([Figure 16.5](#)):

- a. number of blastomeres should be greater or equal to eight
- b. fragmentation of less than 20%
- c. no multi-nucleated blastomeres

116–118 hours post insemination/ICSI ([Figures 16.3 and 16.4](#)):

- a. the blastocoel cavity should be full
- b. ICM should be numerous and tightly packed
- c. trophectoderm cells should be numerous and cohesive

Which of the preceding criteria will prove to be the most important? Or will they all be needed? In brief, AI has refined sequential embryo assessment but still relies on the preceding criteria so as to decipher the most impactful hurdles of development. At every step an embryo is effectively given a positive mark when it reaches the ideal criteria of a certain stage. It would however be possible that an embryo may not pass one step but would pass the hurdle at a following step. The embryo or embryos attaining the best criteria at each

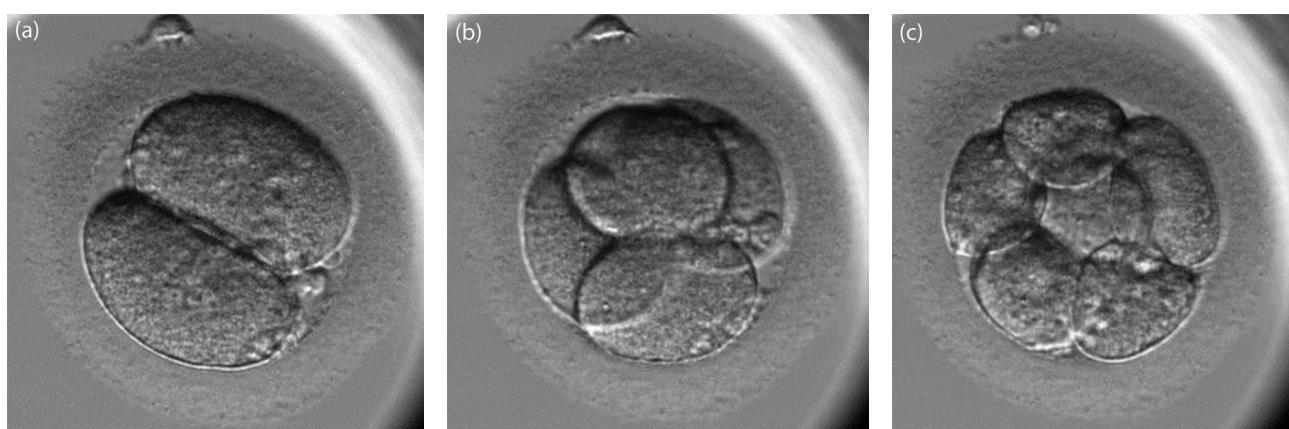


FIGURE 16.5 Ideal features of embryos scored at (a) 25–26 hours, (b) 42–44 hours, and (c) 66–68 hours post insemination/ICSI. For greater details on the scoring criteria, see Sakkas et al. [49], Shoukir et al. [51] and Van Royen et al. [46].

step would therefore be the ones that would be selected for transfer (Figure 16.6). For example, if we are attempting to transfer a single embryo to a patient, the following scenario could be envisaged. An embryo may not pass any of the earlier hurdles but still form a high-grade blastocyst on day 5. If this were the most successful of the cohort of embryos then this would be the one selected. If, however, six blastocysts were observed on day 5, all of equally high grade, then the blastocyst that had achieved the most positive scores at each of the previous hurdles could be transferred. If the shortened protocol was used and only day 2 was the previous score then the best-looking day 2 embryos would be ranked as better. Furthermore, patients who have low numbers of embryos, and will have transfer on day 2 or 3, could be assessed using the initial criteria and the embryo that passed the initial hurdles would be selected. Proposed schedules of embryo selection are given in Figure 16.6, taking into account different strategies or assessment criteria. It is important to note that to-date the strongest criteria of selection appear to be the selection of a high-quality blastocyst on day 5 of development [54, 71]. Since we first developed and advocated this approach to embryo selection in [97] and then developing it further to include a weighted score for each stage [98], such data has been incorporated into many algorithms for use with time-lapse microscopy, to facilitate both embryo deselection and selection.

One of the perennial questions that still remains is whether cleavage or blastocyst transfer is performed. Even though we

have the choice of assessing multiple stages by either repeated manual or time-lapse assessments, many groups have looked at actually minimizing their assessment of embryos and culturing all embryos directly to the blastocyst stage (Figure 16.6). This has coincided with a shift in general opinion that blastocyst morphology can potentially provide stronger evidence of viability [73] and also the data consistently indicating that single embryo transfer is more likely to be implemented with blastocyst rather than cleavage transfer [99, 100]. This would possibly involve scoring fertilization on day 1, and then leaving embryos in culture until day 5, 6, and 7 when they are assessed for transfer or cryopreservation at the blastocyst stage. For example, the clinic may want to set a limit on how many fertilized embryos they need to continue for blastocyst culture (Table 16.1). Data from Boston IVF indicates that a patient <40 years of age with at least four fertilized embryos has over an 80% chance of having a blastocyst for transfer or cryopreservation. Some argue that even identifying blastocysts where the 2PN have not been observed is valuable and that any blastocyst could be included in the pool of usable embryos [101].

The following strategies would be available for most laboratories. Firstly, standard assessment of embryos adopting all or some of the aforementioned morphological criteria. A practical issue for performing such a selection process is that embryos need to be cultured in individual drops. This may remove any necessary benefits of culturing embryos in groups [102–104]. Group culture could

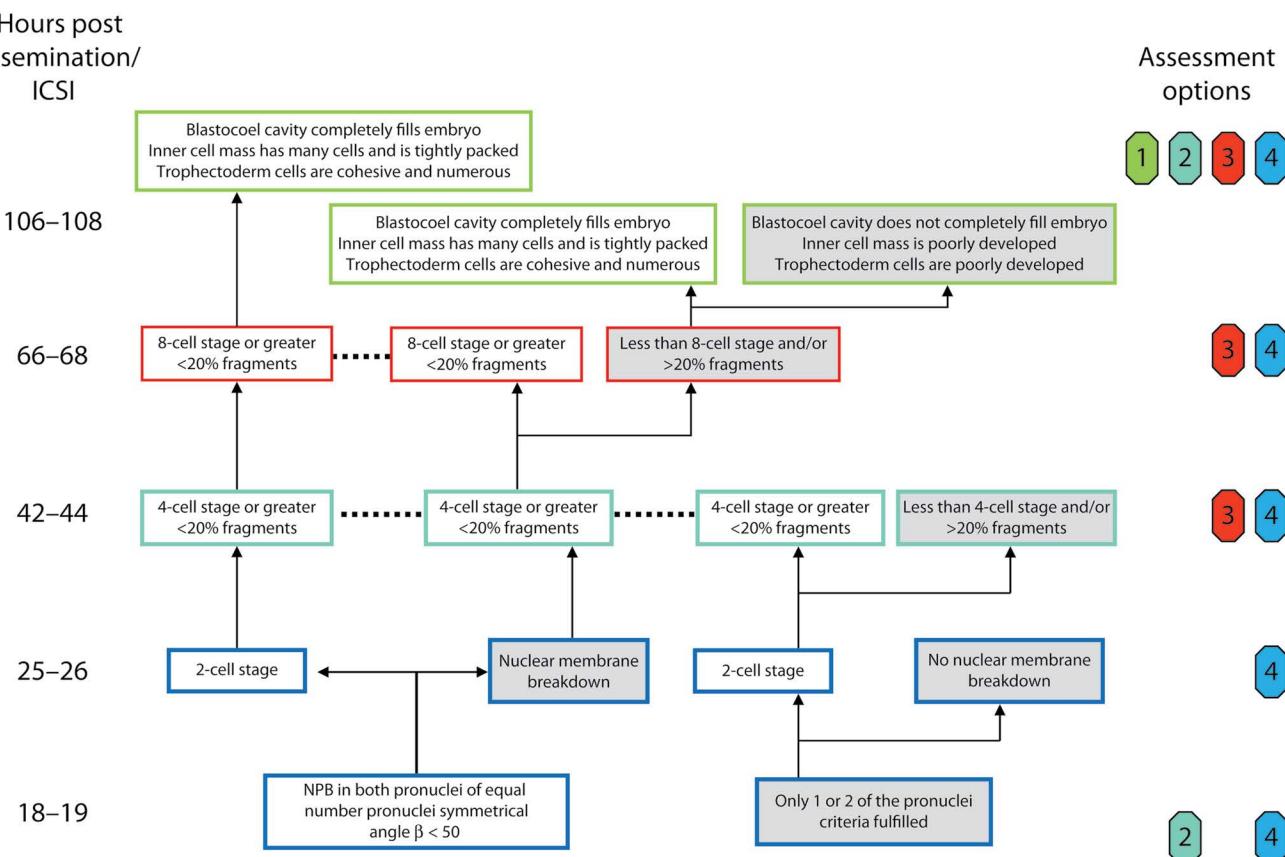


FIGURE 16.6 A strategy for selecting a single embryo for transfer using different morphological assessment options. Number 1 would entail a single assessment only at the blastocyst stages (green); number 2 (light blue) would allow triaging of patients on day 1 followed by assessment at the blastocyst stage; number 3 (red) would allow triaging of patients on day 2 and/or 3 followed by assessment at the blastocyst stage. Number 4 (dark blue) would allow triaging of patients on each day 1, 2, and 3 followed by assessment at the blastocyst stage.

TABLE 16.1 The probability of obtaining at least 1–6 blastocysts for transfer or cryopreservation in relation to having three, four, five, or six fertilized two pronuclear embryos.

	NUMBER OF 2PN FERTILIZED	AT LEAST					
		1	2	3	4	5	6
AGE GROUP		BLASTOCYST(S)					
(YEARS)							
<35	6	96.11%	81.11%	57.96%	35.00%	14.26%	3.15%
	5	94.41%	76.40%	45.41%	8.83%	7.57%	
	4	92.66%	67.09%	37.74%	23.90%		
	3	77.63%	42.98%	11.84%			
35–37	6	91.82%	77.27%	49.32%	26.59%	10.91%	2.05%
	5	92.98%	73.68%	39.91%	17.98%	5.04%	
	4	87.61%	56.88%	22.48%	4.82%		
	3	78.16%	42.32%	11.60%			
38–40	6	93.89%	77.02%	46.70%	21.52%	7.58%	1.71%
	5	89.50%	67.06%	36.04%	14.32%	1.91%	
	4	82.70%	51.08%	19.46%	3.51%		
	3	76.74%	36.86%	9.06%			
	6	82.43%	37.24%	10.88%	3.35%	0.42%	0.00%
>40	5	68.84%	27.90%	6.16%	1.45%	0.36%	
	4	66.06%	24.55%	3.25%	0.36%		
	3	52.63%	14.57%	3.24%			

Note: The data are from an analysis of over 20,000 fertilized embryos left for culture to the blastocyst stage. The green shade shows when the chance is >90% and yellow shade shows when the chance is between 80% and 90%.

be performed, however; as embryos reach certain milestones they could be triaged to a subsequent embryo culture drop. A further practical issue when embryos need to be observed more often is that using a drop culture system under oil with adequate heating control of all microscope stages will greatly reduce pH, osmolality, and temperature fluctuations [105]. Fortunately, the move to commercialize real-time imaging of embryos has now placed multiple embryo assessment procedures closer to a practical reality, removing any concerns related to constant visualization of the embryos away from the incubator [56, 57, 105]. The further development of this type of imaging system is covered in Chapters 17 to 19. However, the scoring regimens described in detail in this chapter will serve all those clinical laboratories that do not have access to time-lapse analysis. A second approach is to minimize observations and culture directly to the blastocyst stage. As mentioned earlier, this can be performed on all patients [106] or on patients that reach certain milestones such as a specific number of fertilized or cleaved embryos (Table 16.1).

It is evident that with improved culture conditions, together with suitable grading systems, it is possible to dramatically increase implantation rates, decrease the number of embryos transferred, and increase the live birth rate. However, this approach raises two issues; if the laboratory in question is not performing blastocyst transfer, then it cannot rely on advanced grading systems, and, secondly, morphology will only tell us a limited amount about the physiological status of the embryo. The rest of this chapter is, therefore, devoted to the application of novel tests of embryonic function. It is assumed that such tests must be non-invasive for the adoption in clinical use. Therefore, methods that can be considered as semi-invasive, i.e. those that involve embryo biopsy prior to cell analysis are not considered here and are discussed in Chapters 13 and 25.

Beyond embryo morphology: The non-invasive quantification of embryo physiology

A number of quantitative techniques have been trialled which attempt to monitor the uptake of specific nutrients by the embryo from the surrounding medium, and to detect the secretion of specific metabolites and factors into the medium (Figure 16.7). Such approaches have strived to measure changes in culture media and fulfil the following three key criteria so that they can be applicable in IVF clinics.

1. They must have the ability to measure the change without damaging the embryo.
2. They must have the ability to measure the change quickly (this requirement may however be lower, as the success of vitrification and move away from fresh transfers [107] may circumvent the need for a rapid test).
3. They must have the ability to measure the change consistently and accurately.

The analysis of metabolite levels within spent embryo culture media fulfils the preceding criteria, and has been one method examined to augment the analysis of embryo morphology as a means of embryo selection. Three approaches have been evaluated: analysis of carbohydrate utilization, the turnover of amino acids, and the analysis of the embryonic metabolome. The first two approaches could be considered analysis of the activity of specific metabolic pathways, whereas analysis of the metabolome should be considered as the systematic analysis of the inventory of metabolites that represent the functional phenotype at the cellular level.

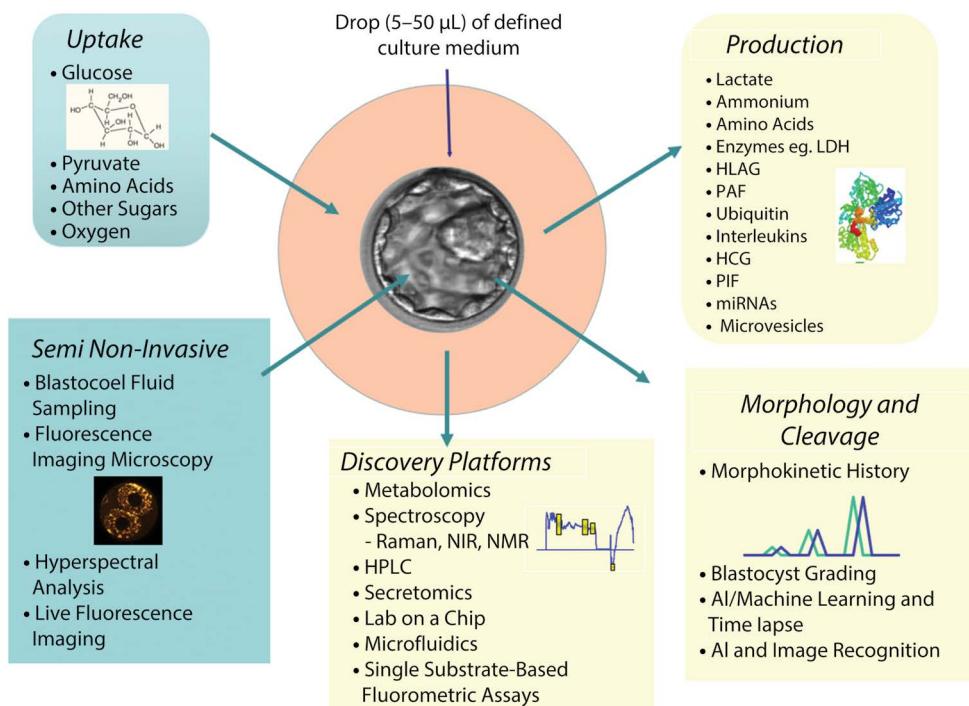


FIGURE 16.7 Options for the non-invasive analysis of human embryo nutrient consumption and metabolite/factor production. Individual blastocysts are incubated in 5.0 to 50.0 μL volumes of defined medium. Serial or end point samples of medium can then be removed for analysis and an indirect measurement of metabolic pathways can be ascertained by measuring uptake or production of various factors or using discovery platforms. Non-invasive platforms can also include current morphology and cleavage criteria using static or time-lapse measurements. Semi-non-invasive platforms are also under development using novel microscopy platforms or examining the blastocoel fluid.

Depending upon the technology employed to analyse the metabolome, one does not necessarily obtain identification of specific metabolites, but rather one is able to create an algorithm that relates to cell function and hence to potential viability.

Analysis of carbohydrate utilization

A relationship between metabolic activity and embryo development and viability has been established over several decades [108]. As early as 1970, Menke and McLaren revealed that mouse blastocysts developed in basic culture conditions lost their ability to oxidize glucose [109]. This initial observation was followed by several studies that elucidated changes in embryo metabolism associated with loss of developmental capacity *in vitro* [reviewed by [110]]. In 1980, Renard et al. [111] observed that day-10 cattle blastocysts which had an elevated glucose uptake developed better, both in culture and *in vivo*, after transfer than those blastocysts with a glucose uptake below this value. In 1987, using the then relatively new technique of non-invasive micro-fluorescence, Gardner and Leese [112] measured glucose uptake by individual day-4 mouse blastocysts prior to transfer to recipient females. Those embryos that went to term had a significantly higher glucose uptake in culture than those embryos that failed to develop after transfer. This work was then built on by Lane and Gardner [113], who showed that glycolytic rate of mouse blastocysts could be used to select embryos for transfer prospectively. Morphologically identical mouse blastocysts with equivalent diameters were identified, using metabolic criteria, as “viable” prior to transfer and had a fetal development of 80%. In contrast, those embryos that exhibited an abnormal metabolic profile (compared to *in vivo* developed controls) developed at a rate of only 6%. Clearly, such data

provides dramatic evidence that metabolic function is linked to embryo viability (Figures 16.8a and b), and that perturbations in relative activity of metabolic pathways is associated with loss of cell function, leading to compromised development post transfer.

Analysis of the relationship between human embryo nutrition and subsequent development *in vitro* was undertaken by Gardner et al. [114], who determined that glucose consumption on day 4 by human embryos was twice as high in those embryos that went on to form blastocysts. Subsequently, Gardner and colleagues [115] went on to confirm a positive relationship between glucose uptake and human embryo viability on day 4 and day 5 of development (Figure 16.9). Furthermore, the data generated indicate that differences in nutrient utilization differ between male and female embryos, a phenomenon previously documented in other mammalian species [116, 117]. A subsequent analysis of more than 200 human blastocysts again confirmed that those embryos that go on to form a pregnancy consume significantly more glucose than those blastocysts which failed [118].

Currently making accurate analysis of nutrient uptake by individual embryos is performed using non-commercial fluorescence assays, which have been limited to just a few laboratories worldwide. The widespread implementation and subsequent validation of this approach should be made possible through the development of chip-based devices capable of quantitation accurately sub-microlitre volumes of medium [119–121].

Analysis of amino acid utilization

In studies on amino acid turnover by human embryos, Houghton et al. [122] determined that alanine release into the surrounding medium on day 2 and day 3 was highest in those embryos that did

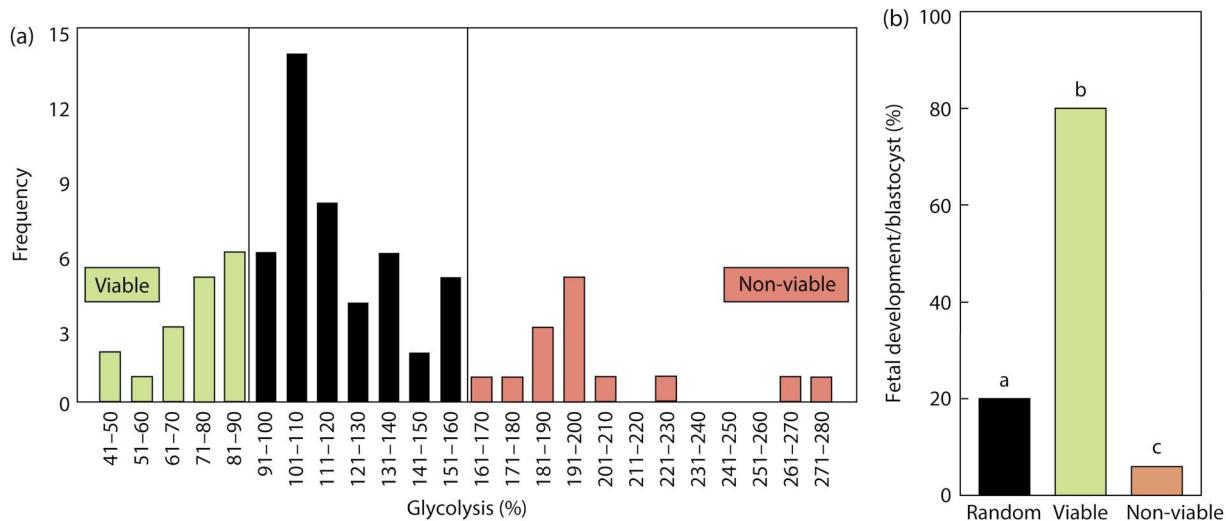


FIGURE 16.8 (a) Distribution of glycolytic activity in a population of 79 morphologically similar mouse blastocysts cultured in medium DM1. The lowest 15% of glycolytic activity (<88%) were considered viable, while the highest 15% of the range (>160%) were deemed non-viable. (Adapted from [113].) (b) Fetal development of mouse blastocysts selected for transfer according to whether they were considered viable or non-viable using glycolytic activity as a biochemical marker. On each day of the experiment, a selection of blastocysts was transferred at random, along with those selected as either viable or non-viable. Different superscripts ^{a,b,c} indicate significantly different populations ($P < 0.01$).

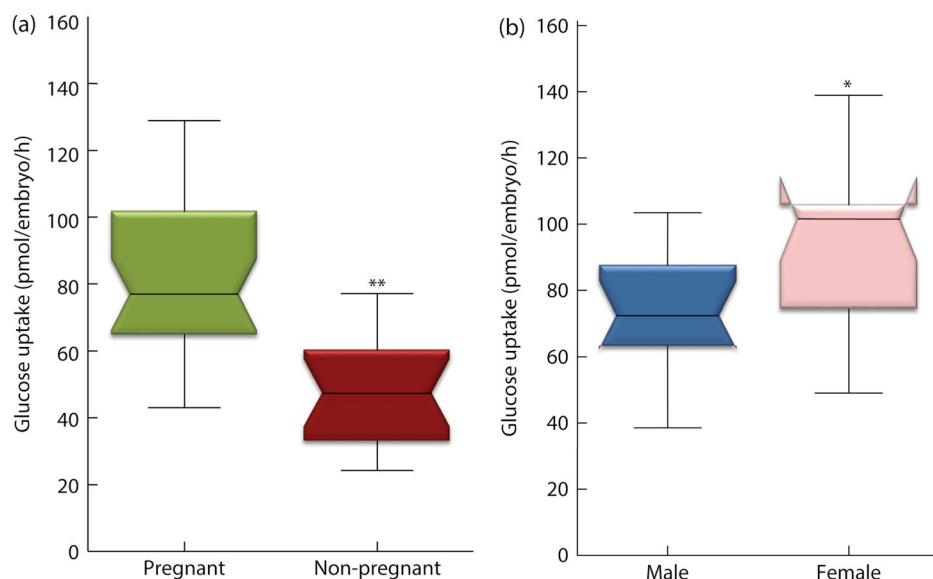


FIGURE 16.9 Relationship between glucose consumption on day 4 of development and human embryo viability and embryo sex. (a) Glucose uptake on day 4 of embryonic development and pregnancy outcome (positive fetal heart beat). Notches represent the confidence interval of the median, and the depth of the box represents the interquartile range (50% of the data); whiskers represent the 5% and 95% quartiles. The line across the box is the median glucose consumption. **, significantly different from pregnant ($P < 0.01$). (b) Glucose uptake by male and female embryos on day 4 of development. Notches represent the confidence interval of the median, and the depth of the box represents the interquartile range (50% of the data); whiskers represent the 5% and 95% quartiles. The line across the box is the median glucose consumption. *, significantly different from male embryos ($P < 0.05$). (Adapted from [115].)

not form blastocysts. Subsequently, Brison et al. [123] reported changes in concentration of amino acids in the spent medium of human zygotes cultured for 24 hours in an embryo culture medium containing a mixture of amino acids using high performance liquid chromatography. It was found that asparagine, glycine, and leucine utilized in the 24 hours following fertilization

were significantly associated with clinical pregnancy and live birth following day 2 embryo transfer. Further analysis also revealed an association with aneuploidy and embryonic sex with amino acid turnover [124]. Recent works have further revealed that amino acid consumption by human blastocysts is related to their grade, morphokinetics, and AI ranking [118].

Ongoing studies in this area could help to identify which amino acids at each stage of development is linked with subsequent viability. Recent animal studies have revealed how dynamic the use of amino acids is and how uptake can be affected by other aspects of the culture system, such as oxygen and the accumulation of ammonium through the spontaneous breakdown and metabolism of amino acids [125, 126]. Consequently, data on the use of nutrients needs to be carefully interpreted with regards to the conditions under which the embryos were developed.

Metabolomics

Evolving metabolomics technologies may allow us in the future to measure multiple factors in embryo culture media. Initial and encouraging metabolic studies of embryos indicated that embryos that result in pregnancy are different in their metabolic profile compared to embryos that do not lead to pregnancies [127]. Investigation of the metabolome of embryos, as detected in the culture media they grow in, using targeted spectroscopic analysis and bioinformatics did show differences in some initial proof of principle studies [127].

Although a series of preliminary studies [128–131] showed a benefit of metabolomics-related techniques, they were largely based on retrospective studies and performed in a single research laboratory as distinct from a real clinical setting. The subsequent randomized clinical trials comparing standard morphological techniques for embryo selection versus using the near infrared (NIR) system to rank embryos within a cohort that had good morphology and were being selected for either transfer or cryopreservation failed to show compelling benefits [128, 131]. More recently, Pais et al. [132] examined the secretome of human embryos using Matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF) mass spectrometry. They applied bioinformatic analysis to identify specific spectra of euploid and aneuploid embryo secretome signatures and were able to differentiate between genotypes with a sensitivity of 84% and a false positive rate of 18%.

Similarly, although Katz-Jaffe et al. [133–135] revealed that the proteome of individual human blastocysts of the same grade differed between embryos, and also identified a number of secreted protein markers that could be used to identify the best embryo, a relationship between such biomarkers and subsequent viability has yet to be validated prospectively.

Although the use of metabolomic and proteomic platforms has yet to be proven and employed clinically, analysis of embryo function for its own sake can greatly enhance our understanding of embryo development and hence such approaches could ultimately assist in defining parameters than can be used in embryo selection. To this end, an analysis of the relationship between the morphokinetics development of embryos and their metabolic activity has been undertaken.

When morphometrics and metabolic analysis collide

Using a mouse model to analyse the relationship between key morphometric and metabolic data from individual IVF-derived embryos, Lee and colleagues determined that blastocysts developing from those embryos exhibiting early cleavage (and hence presumed to have a higher viability) possessed a metabolic profile of increased glucose uptake and reduced rates of glycolysis (and hence exhibiting metabolic characteristics of enhanced viability) [136]. Furthermore, it was observed that blastocysts

developed from embryos with early cleavage also consumed more aspartate, potentially reflecting a more active malate-aspartate shuttle, which has been implicated in the regulation of blastocyst metabolism and viability [137, 138]. Together, such data generate renewed excitement regarding the potential of non-invasive quantification of embryo physiology to assist in the selection of the most viable embryo for transfer, and the potential of combining two independent means of assessing the pre-implantation embryo to improve the accuracy of selection.

Non-invasive fluorescence microscopy

In recent years a greater focus has been made on implementation of fluorescence microscopy to investigate embryo metabolism. Two techniques have been examined, Fluorescence Lifetime Imaging Microscopy (FLIM) and Hyperspectral Microscopy. Both technologies rely on autofluorescence, which is the natural emission of light by biological structures such as mitochondria and lysosomes. The most commonly observed autofluorescing molecules are NADPH and flavins; the extracellular matrix can also contribute to autofluorescence because of the intrinsic properties of collagen and elastin. Using FLIM, it was determined [139] that the metabolic state of human blastocysts continuously varies over time. Although it was possible to identify metabolic variations between blastocysts in relation to day of development and developmental expansion stage, their morphological grade was not related to metabolic state. Interestingly, substantial metabolic variations between blastocysts from the same patients were observed. Furthermore, there was significant metabolic heterogeneity within individual blastocysts, including between the ICM and the trophectoderm. Both FLIM and hyperspectral microscopy have also provided evidence that they can distinguish between euploid and aneuploid embryos [140, 141].

The non-invasive nature of both these microscopy techniques and the aforementioned observations combined give merit to the intricate timing of metabolic shifts in human embryos and how understanding these changes has great potential to improve our ability to distinguish between viable and non-viable embryos.

Other specific factors

Other techniques have also been reported to measure metabolic parameters in culture media; however, they have yet to be tested in a clinical IVF setting. These include the self-referencing electrophysiological technique, which is a non-invasive measurement of the physiology of individual cells and monitors the movement of ions and molecules between the cell and the surrounding media [142, 143]. Another technique using a probe was initially developed by Unisense to non-invasively measure oxygen consumption of developing embryos. Interestingly, although this technology was shown to correlate with bovine blastocyst development it was less successful in predicting mouse embryo development [144, 145].

A number of studies have also investigated the assessment of secreted factors in the embryo culture media (Figure 16.7) and correlated them with better embryo development and pregnancy rates. One such factor is soluble HLA-G [146, 147], which is believed to protect the developing embryo from destruction by the maternal immune response. Soluble HLA-G has been found in media surrounding the early embryo and a number of papers have also reported that its presence correlates with the improved pregnancy potential of an embryo [148–150]. However,

some studies have raised some serious concerns regarding the use of HLA-G production as a marker of further developmental potential [151–153], and prospective clinical trials are needed to further evaluate this parameter. Included in the studies examining secretion of factors in the media by embryos are numerous papers examining the secretion of platelet-activating factor (PAF). The clinical utility of PAF in an IVF setting has also yet to be stringently examined (see review by O'Neill [154]). Other factors are currently under investigation, including one called the pre-implantation factor (PIF), which has been reported to provide some indication of embryo viability when measured and to possibly improve embryo quality when placed in embryo culture media [155]. Numerous other candidates have also been postulated and tested, including human chorionic gonadotropin [156, 157] and interleukin 6 [158]. All these molecules could also benefit from novel single substrate-based fluorometric assays that are currently being optimized [159].

It is beyond doubt that markers do exist in the spent embryo culture media indicative of viability. The most advanced current techniques are AI coupled with time-lapse or static blastocyst images (see Chapters 18 and 19) and the analysis of cell-free DNA in embryo culture media [160] to assess chromosomal copy numbers. The major benefits of a non-invasive fluorescence technology is the fact that the technology visualizes the whole embryos, and the time taken to assess the samples is relatively short, making it possible to perform the analysis just prior to fresh embryo transfer or freezing. Many research groups around the world are still attempting to make this a reality for the IVF clinic.

Summary

Analysis of embryo morphology and the development of suitable grading systems have greatly assisted in the selection of human embryos for transfer. However, it is proposed that in the near future embryo selection will also be significantly aided by the non-invasive analysis of embryo physiology and function, using approaches that better quantify embryo metabolism. The addition of such technologies will be of immense value in helping both clinicians and embryologists to more confidently select the most viable embryos within a cohort and making the need to transfer more than one embryo a thing of the past.

References

- Mansour R, Ishihara O, Adamson GD, Dyer S, de Mouzon J, Nygren KG, et al. International Committee for Monitoring Assisted Reproductive Technologies world report: Assisted Reproductive Technology 2006. *Hum Reprod.* 2014;29(7):1536–51.
- Chambers GM, Dyer S, Zegers-Hochschild F, de Mouzon J, Ishihara O, Banker M, et al. International Committee for Monitoring Assisted Reproductive Technologies world report: Assisted Reproductive technology, 2014. *Hum Reprod.* 2021;36(11):2921–34.
- De Geyter C, Wyns C, Calhaz-Jorge C, de Mouzon J, Ferraretti AP, Kupka M, et al. 20 years of the European IVF-monitoring Consortium registry: What have we learned? A comparison with registries from two other regions. *Hum Reprod.* 2020;35(12):2832–49.
- Fauser BC. Towards the global coverage of a unified registry of IVF outcomes. *Reprod Biomed Online.* 2019;38(2):133–7.
- de Mouzon J, Goossens V, Bhattacharya S, Castilla JA, Ferraretti AP, Korsak V, et al. Assisted reproductive technology in Europe, 2006: Results generated from European registers by ESHRE. *Hum Reprod.* 2010;25(8):1851–62.
- ANZARD data [cited 2022 December 6]. Available from: https://npesu.unsw.edu.au/sites/default/files/npesu/data_collection/Assisted%20Reproductive%20Technology%20in%20Australia%20and%20New%20Zealand%202020.pdf.
- Nyboe Andersen A, Goossens V, Bhattacharya S, Ferraretti AP, Kupka MS, de Mouzon J, et al. Assisted reproductive technology and intrauterine inseminations in Europe, 2005: Results generated from European registers by ESHRE: ESHRE. The European IVF Monitoring Programme (EIM), for the European Society of Human Reproduction and Embryology (ESHRE). *Hum Reprod.* 2009;24(6):1267–87.
- SART. [cited 2022 December 4]. Available from: https://www.sartcoronline.com/rptCSR_PublicMultYear.aspx?reportingYear=2019.
- Adashi EY, Barri PN, Berkowitz R, Braude P, Bryan E, Carr J, et al. Infertility therapy-associated multiple pregnancies (births): An ongoing epidemic. *Reprod Biomed Online.* 2003;7(5):515–42.
- Bromer JG, Ata B, Seli M, Lockwood CJ, Seli E. Preterm deliveries that result from multiple pregnancies associated with assisted reproductive technologies in the USA: A cost analysis. *Curr Opin Obstet Gynecol.* 2011;23(3):168–73.
- Collins J. Cost efficiency of reducing multiple births. *Reprod Biomed Online.* 2007;15(Suppl 3):35–9.
- Gerris J, De Sutter P, De Neubourg D, Van Royen E, Vander Elst J, Mangelschots K, et al. A real-life prospective health economic study of elective single embryo transfer versus two-embryo transfer in first IVF/ICSI cycles. *Hum Reprod.* 2004;19(4):917–23.
- Ledger WL, Anumba D, Marlow N, Thomas CM, Wilson EC. The costs to the NHS of multiple births after IVF treatment in the UK. *BJOG.* 2006;113(1):21–5.
- Practice Committee of the American Society for Reproductive Medicine and the Practice Committee for the Society for Assisted Reproductive Technologies. Guidance on the limits to the number of embryos to transfer: A committee opinion. *Fertil Steril.* 2021;116(3):651–4.
- Hu L, Bu Z, Huang G, Sun H, Deng C, Sun Y. Assisted reproductive technology in China: Results generated from data reporting system by CSR from 2013 to 2016. *Front Endocrinol (Lausanne).* 2020;11:458.
- Inge GB, Brinsden PR, Elder KT. Oocyte number per live birth in IVF: Were Steptoe and Edwards less wasteful? *Hum Reprod.* 2005;20(3):588–92.
- Patrizio P, Sakkas D. From oocyte to baby: A clinical evaluation of the biological efficiency of in vitro fertilization. *Fertil Steril.* 2009;91(4):1061–6.
- Law YJ, Zhang N, Venetis CA, Chambers GM, Harris K. The number of oocytes associated with maximum cumulative live birth rates per aspiration depends on female age: A population study of 221 221 treatment cycles. *Hum Reprod.* 2019;34(9):1778–87.
- Vaughan DA, Leung A, Resetkova N, Ruthazer R, Penzias AS, Sakkas D, et al. How many oocytes are optimal to achieve multiple live births with one stimulation cycle? The one-and-done approach. *Fertil Steril.* 2017;107(2):397–404 e3.
- Blesa D, Ruiz-Alonso M, Simón C. Clinical management of endometrial receptivity. *Semin Reprod Med.* 2014;32(5):410–3.
- Edgell TA, Rombauts LJ, Salamonsen LA. Assessing receptivity in the endometrium: The need for a rapid, non-invasive test. *Reprod Biomed Online.* 2013;27(5):486–96.
- Evans J, Hannan NJ, Hincks C, Rombauts LJ, Salamonsen LA. Defective soil for a fertile seed? Altered endometrial development is detrimental to pregnancy success. *PLoS One.* 2012;7(12):e53098.
- Miravet-Valenciano JA, Rincon-Bertolin A, Vilella F, Simon C. Understanding and improving endometrial receptivity. *Curr Opin Obstet Gynecol.* 2015;27(3):187–92.
- Salamonsen LA, Evans J, Nguyen HP, Edgell TA. The microenvironment of human implantation: Determinant of reproductive success. *Am J Reprod Immunol.* 2016;75(3):218–25.

25. Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF. A formula for scoring human embryo growth rates in vitro fertilization: Its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *J In Vitro Fert Embryo Transf.* 1986;3(5):284–95.
26. Edwards RG, Fishel SB, Cohen J, Fehilly CB, Purdy JM, Slater JM, et al. Factors influencing the success of in vitro fertilization for alleviating human infertility. *J In Vitro Fert Embryo Transf.* 1984;1(1):3–23.
27. De Neubourg D, Gerris J. Single embryo transfer – state of the art. *Reprod Biomed Online.* 2003;7(6):615–22.
28. Sakkas D, Gardner DK. Noninvasive methods to assess embryo quality. *Curr Opin Obstet Gynecol.* 2005;17(3):283–8.
29. Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: A prospective randomized trial. *Fertil Steril.* 2004;81(3):551–5.
30. Gardner DK, Balaban B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICs': Is looking good still important? *Mol Hum Reprod.* 2016;22(10):704–18.
31. Gardner DK, Meseguer M, Rubio C, Treff NR. Diagnosis of human preimplantation embryo viability. *Hum Reprod Update.* 2015;21(6):727–47.
32. Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature.* 1988;332(6163):459–61.
33. Scott L, Alvero R, Leondires M, Miller B. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. *Hum Reprod.* 2000;15(11):2394–403.
34. Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. *Hum Reprod.* 1999;14(5):1318–23.
35. Gardner RL. The early blastocyst is bilaterally symmetrical and its axis of symmetry is aligned with the animal-vegetal axis of the zygote in the mouse. *Development.* 1997;124(2):289–301.
36. Antczak M, Van Blerkom J. Oocyte influences on early development: The regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Mol Hum Reprod.* 1997;3(12):1067–86.
37. Antczak M, Van Blerkom J. Temporal and spatial aspects of fragmentation in early human embryos: Possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. *Hum Reprod.* 1999;14(2):429–47.
38. Garello C, Baker H, Rai J, Montgomery S, Wilson P, Kennedy CR, et al. Pronuclear orientation, polar body placement, and embryo quality after intracytoplasmic sperm injection and in-vitro fertilization: Further evidence for polarity in human oocytes? *Hum Reprod.* 1999;14(10):2588–95.
39. Coticchio G, Guglielmo MC, Albertini DF, Dal Canto M, Mignini Renzini M, De Ponti E, et al. Contributions of the actin cytoskeleton to the emergence of polarity during maturation in human oocytes. *Mol Hum Reprod.* 2014;20(3):200–7.
40. Scott LA, Smith S. The successful use of pronuclear embryo transfers the day following oocyte retrieval. *Hum Reprod.* 1998;13(4):1003–13.
41. Scott L. Pronuclear scoring as a predictor of embryo development. *Reprod Biomed Online.* 2003;6(2):201–14.
42. Aguilar J, Motato Y, Escribá MJ, Ojeda M, Muñoz E, Meseguer M. The human first cell cycle: Impact on implantation. *Reprod Biomed Online.* 2014;28(4):475–84.
43. Cruz M, Garrido N, Gadea B, Muñoz M, Pérez-Cano I, Meseguer M. Oocyte insemination techniques are related to alterations of embryo developmental timing in an oocyte donation model. *Reprod Biomed Online.* 2013;27(4):367–75.
44. Ezoe K, Coticchio G, Takenouchi H, Taoda S, Namerikawa S, Honda K, et al. Spatiotemporal perturbations of pronuclear breakdown preceding syngamy affect early human embryo development: A retrospective observational study. *J Assist Reprod Genet.* 2022;39(1):75–84.
45. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de Meerssche M, Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: A prospective randomized clinical trial. *Hum Reprod.* 1999;14(10):2581–7.
46. Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, et al. Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod.* 1999;14(9):2345–9.
47. De Neubourg D, Gerris J, Mangelschots K, Van Royen E, Vercruyssen M, Elseviers M. Single top quality embryo transfer as a model for prediction of early pregnancy outcome. *Hum Reprod.* 2004;19(6):1476–9.
48. Bavister BD. Culture of preimplantation embryos: Facts and artifacts. *Hum Reprod Update.* 1995;1(2):91–148.
49. Sakkas D, Percival G, D'Arcy Y, Sharif K, Afnan M. Assessment of early cleaving in vitro fertilized human embryos at the 2-cell stage before transfer improves embryo selection. *Fertil Steril.* 2001;76(6):1150–6.
50. Sakkas D, Shoukir Y, Chardonnens D, Bianchi PG, Campana A. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. *Hum Reprod.* 1998;13(1):182–7.
51. Shoukir Y, Campana A, Farley T, Sakkas D. Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: A novel indicator of embryo quality and viability. *Hum Reprod.* 1997;12(7):1531–6.
52. Salumets A, Hydén-Granskog C, Mäkinen S, Suikkari AM, Tüttinen A, Tuuri T. Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Hum Reprod.* 2003;18(4):821–5.
53. Van Montfoort AP, Dumoulin JC, Kester AD, Evers JL. Early cleavage is a valuable addition to existing embryo selection parameters: A study using single embryo transfers. *Hum Reprod.* 2004;19(9):2103–8.
54. Guerif F, Le Gouge A, Giraudieu B, Poindron J, Bidault R, Gasnier O, et al. Limited value of morphological assessment at days 1 and 2 to predict blastocyst development potential: A prospective study based on 4042 embryos. *Hum Reprod.* 2007;22(7):1973–81.
55. Neuber E, Rinaudo P, Trimarchi JR, Sakkas D. Sequential assessment of individually cultured human embryos as an indicator of subsequent good quality blastocyst development. *Hum Reprod.* 2003;18(6):1307–12.
56. Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remohí J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod.* 2011;26(10):2658–71.
57. Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, et al. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol.* 2010;28(10):1115–21.
58. Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boosanfar R, et al. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: Results from a prospective multicenter trial. *Fertil Steril.* 2013;100(2):412–9 e5.
59. Herrero J, Meseguer M. Selection of high potential embryos using time-lapse imaging: The era of morphokinetics. *Fertil Steril.* 2013;99(4):1030–4.
60. Ebner T, Moser M, Shebl O, Sommergruber M, Gaiswinkler U, Tews G. Morphological analysis at compacting stage is a valuable prognostic tool for ICSI patients. *Reprod Biomed Online.* 2009;18(1):61–6.

61. Feil D, Henshaw RC, Lane M. Day 4 embryo selection is equal to Day 5 using a new embryo scoring system validated in single embryo transfers. *Hum Reprod.* 2008;23(7):1505–10.
62. The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: Proceedings of an expert meeting. *Reprod Biomed Online.* 2012;25(2):146–67.
63. Cobo A, de los Santos MJ, Castellò D, Gámiz P, Campos P, Remohí J. Outcomes of vitrified early cleavage-stage and blastocyst-stage embryos in a cryopreservation program: Evaluation of 3,150 warming cycles. *Fertil Steril.* 2012;98(5):1138–46 e1.
64. Sparks AE. Human embryo cryopreservation-methods, timing, and other considerations for optimizing an embryo cryopreservation program. *Semin Reprod Med.* 2015;33(2):128–44.
65. Forman EJ, Hong KH, Treff NR, Scott RT. Comprehensive chromosome screening and embryo selection: Moving toward single euploid blastocyst transfer. *Semin Reprod Med.* 2012;30(3):236–42.
66. Schoolcraft WB, Treff NR, Stevens JM, Ferry K, Katz-Jaffe M, Scott RT Jr. Live birth outcome with trophectoderm biopsy, blastocyst vitrification, and single-nucleotide polymorphism microarray-based comprehensive chromosome screening in infertile patients. *Fertil Steril.* 2011;96(3):638–40.
67. Scott RT Jr, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, et al. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: A randomized controlled trial. *Fertil Steril.* 2013;100(3):697–703.
68. Treff NR, Forman EJ, Scott RT Jr. Next-generation sequencing for preimplantation genetic diagnosis. *Fertil Steril.* 2013;99(6):e17–8.
69. Gardner DK, Schoolcraft WB. In vitro culture of human blastocysts. In: *Towards Reproductive Certainty: Infertility and Genetics Beyond*. Carnforth, Parthenon Press. Jansen R, Mortimer D (eds.). pp 378–388 1999.
70. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Hum Reprod.* 2011;26(6):1270–83.
71. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertil Steril.* 2000;73(6):1155–8.
72. Gardner DK, Stevens J, Sheehan CB, Schoolcraft WB. Morphological assessment of the human blastocyst. In: *Human Preimplantation Embryo Selection*. Elder K, Cohen J (eds.). London, Informa Healthcare. pp. 79–87, 2007.
73. Ahlström A, Westin C, Reismer E, Wiklund M, Hardarson T. Trophectoderm morphology: An important parameter for predicting live birth after single blastocyst transfer. *Hum Reprod.* 2011;26(12):3289–96.
74. Chen X, Zhang J, Wu X, Cao S, Zhou L, Wang Y, et al. Trophectoderm morphology predicts outcomes of pregnancy in vitrified-warmed single-blastocyst transfer cycle in a Chinese population. *J Assist Reprod Genet.* 2014;31(11):1475–81.
75. Hill MJ, Richter KS, Heitmann RJ, Graham JR, Tucker MJ, DeCherney AH, et al. Trophectoderm grade predicts outcomes of single-blastocyst transfers. *Fertil Steril.* 2013;99(5):1283–9 e1.
76. Honnma H, Baba T, Sasaki M, Hashiba Y, Ohno H, Fukunaga T, et al. Trophectoderm morphology significantly affects the rates of ongoing pregnancy and miscarriage in frozen-thawed single-blastocyst transfer cycle in vitro fertilization. *Fertil Steril.* 2012;98(2):361–7.
77. Thompson SM, Onwubalili N, Brown K, Jindal SK, McGovern PG. Blastocyst expansion score and trophectoderm morphology strongly predict successful clinical pregnancy and live birth following elective single embryo blastocyst transfer (eSET): A national study. *J Assist Reprod Genet.* 2013;30(12):1577–81.
78. van der Weiden RM. Trophectoderm morphology grading reflects interactions between embryo and endometrium. *Fertil Steril.* 2013;100(4):e23.
79. Van den Abbeel E, Balaban B, Ziebe S, Lundin K, Cuesta MJ, Klein BM, et al. Association between blastocyst morphology and outcome of single-blastocyst transfer. *Reprod Biomed Online.* 2013;27(4):353–61.
80. Licciardi F, McCaffrey C, Oh C, Schmidt-Sarosi C, McCulloh DH. Birth weight is associated with inner cell mass grade of blastocysts. *Fertil Steril.* 2015;103(2):382–7 e2.
81. Ansell JD, Snow MH. The development of trophoblast in vitro from blastocysts containing varying amounts of inner cell mass. *J Embryol Exp Morphol.* 1975;33(1):177–85.
82. Snow MH, Aitken J, Ansell JD. Role of the inner cell mass in controlling implantation in the mouse. *J Reprod Fertil.* 1976;48(2):403–4.
83. Gardner RL, Johnson MH. An investigation of inner cell mass and trophoblast tissues following their isolation from the mouse blastocyst. *J Embryol Exp Morphol.* 1972;28(2):279–312.
84. Hu KL, Zheng X, Hunt S, Li X, Li R, Mol BW. Blastocyst quality and perinatal outcomes in women undergoing single blastocyst transfer in frozen cycles. *Hum Reprod Open.* 2021;2021(4):hoab036.
85. Kemper JM, Liu Y, Afnan M, Hammond ER, Morbeck DE, Mol BWJ. Should we look for a low-grade threshold for blastocyst transfer? A scoping review. *Reprod Biomed Online.* 2021;42(4):709–16.
86. Fransasiak JM, Forman EJ, Patounakis G, Hong KH, Werner MD, Upham KM, et al. Investigating the impact of the timing of blastulation on implantation: Management of embryo-endometrial synchrony improves outcomes. *Hum Reprod Open.* 2018;2018(4):hoy022.
87. Kroener L, Ambartsumyan G, Britton-Jones C, Dumesic D, Surrey M, Munne S, et al. The effect of timing of embryonic progression on chromosomal abnormality. *Fertil Steril.* 2012;98(4):876–80.
88. Shear MA, Vaughan DA, Modest AM, Seidler EA, Leung AQ, Hacker MR, et al. Blasts from the past: Is morphology useful in PGT-A tested and untested frozen embryo transfers? *Reprod Biomed Online.* 2020;41(6):981–9.
89. Hammond ER, Cree LM, Morbeck DE. Should extended blastocyst culture include Day 7? *Hum Reprod.* 2018;33(6):991–7.
90. Tiegs AW, Sun L, Patounakis G, Scott RT. Worth the wait? Day 7 blastocysts have lower euploidy rates but similar sustained implantation rates as Day 5 and Day 6 blastocysts. *Hum Reprod.* 2019;34(9):1632–9.
91. Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Thornton S. Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. *Reprod Biomed Online.* 2013;27(2):140–6.
92. Rienzi L, Capalbo A, Stoppa M, Romano S, Maggiulli R, Albricci L, et al. No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: A longitudinal cohort study. *Reprod Biomed Online.* 2015;30(1):57–66.
93. Chavez-Badiola A, Flores-Saifie-Farías A, Mendizabal-Ruiz G, Drakeley AJ, Cohen J. Embryo Ranking Intelligent Classification Algorithm (ERICA): Artificial intelligence clinical assistant predicting embryo ploidy and implantation. *Reprod Biomed Online.* 2020;41(4):585–93.
94. Tran NH, Qiao R, Xin L, Chen X, Liu C, Zhang X, et al. Deep learning enables de novo peptide sequencing from data-independent-acquisition mass spectrometry. *Nat Methods.* 2019;16(1):63–6.
95. Basile N, Vime P, Florensa M, Aparicio Ruiz B, García Velasco JA, Remohí J, et al. The use of morphokinetics as a predictor of implantation: A multicentric study to define and validate an algorithm for embryo selection. *Hum Reprod.* 2015;30(2):276–83.
96. Fisch JD, Rodriguez H, Ross R, Overby G, Sher G. The Graduated Embryo Score (GES) predicts blastocyst formation and pregnancy rate from cleavage-stage embryos. *Hum Reprod.* 2001;16(9):1970–5.
97. Sakkas D. Evaluation of embryo quality: A strategy for sequential analysis of embryo development with the aim of single embryo transfer. In: *Textbook of Assisted Reproductive Techniques*. Gardner DK, Weissman A, Howles C, Shoham Z (eds.). London: CRC Press, 2001.

98. Gardner DK, Sakkas D. Assessment of embryo viability: The ability to select a single embryo for transfer—a review. *Placenta.* 2003;24(Suppl B):S5–12.
99. Blake DA, Proctor M, Johnson NP. The merits of blastocyst versus cleavage stage embryo transfer: A Cochrane review. *Hum Reprod.* 2004;19(4):795–807.
100. Glujsovsky D, Quinteiro Retamar AM, Alvarez Sedo CR, Ciapponi A, Cornelisse S, Blake D. Cleavage-stage versus blastocyst-stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev.* 2022;5(5):CD002118.
101. Doody KJ. The time has come to reevaluate the fertilization check. *Fertil Steril.* 2021;115(1):74–5.
102. Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: Amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod.* 1994;50(2):390–400.
103. Lane M, Gardner DK. Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. *Hum Reprod.* 1992;7(4):558–62.
104. Wiley LM, Yamami S, Van Muyden D. Effect of potassium concentration, type of protein supplement, and embryo density on mouse preimplantation development in vitro. *Fertil Steril.* 1986;45(1):111–9.
105. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update.* 2016;22(1):2–22.
106. Marek D, Langley M, Gardner DK, Confer N, Doody KM, Doody KJ. Introduction of blastocyst culture and transfer for all patients in an in vitro fertilization program. *Fertil Steril.* 1999;72(6):1035–40.
107. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C. Freeze-all can be a superior therapy to another fresh cycle in patients with prior fresh blastocyst implantation failure. *Reprod Biomed Online.* 2014;29(3):286–90.
108. Bowman P, McLaren A. Viability and growth of mouse embryos after in vitro culture and fusion. *J Embryol Exp Morphol.* 1970;23(3):693–704.
109. Menke TM, McLaren A. Mouse blastocysts grown in vivo and in vitro: Carbon dioxide production and trophoblast outgrowth. *J Reprod Fertil.* 1970;23(1):117–27.
110. Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology.* 1998;49(1):83–102.
111. Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. *J Reprod Fertil.* 1980;58(1):161–4.
112. Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *J Exp Zool.* 1987;242(1):103–5.
113. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod.* 1996;11(9):1975–8.
114. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril.* 2001;76(6):1175–80.
115. Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod.* 2011;26(8):1981–6.
116. Gardner DK, Larman MG, Thouas GA. Sex-related physiology of the preimplantation embryo. *Mol Hum Reprod.* 2010;16(8):539–47.
117. Sturmy RG, Bermijo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. Amino acid metabolism of bovine blastocysts: A biomarker of sex and viability. *Mol Reprod Dev.* 2010;77(3):285–96.
118. Ferrick L, Lee YSL, Gardner DK. Metabolic activity of human blastocysts correlates with their morphokinetics, morphological grade, KIDSscore and artificial intelligence ranking. *Hum Reprod.* 2020;35(9):2004–16.
119. Swain JE, Lai D, Takayama S, Smith GD. Thinking big by thinking small: Application of microfluidic technology to improve ART. *Lab Chip.* 2013;13(7):1213–24.
120. Urbanski JP, Johnson MT, Craig DD, Potter DL, Gardner DK, Thorsen T. Noninvasive metabolic profiling using microfluidics for analysis of single preimplantation embryos. *Anal Chem.* 2008;80(17):6500–7.
121. Thouas GA, Potter DL, Gardner DK. Microfluidic device for the analysis of gamete and embryo physiology. In: *Human Gametes and Preimplantation Embryos: Assessment and Diagnosis.* Gardner DK, Seli E, Wells D, Sakkas D, (eds.). New York: Springer, pp. 281–99, 2013.
122. Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, et al. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod.* 2002;17(4):999–1005.
123. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, et al. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod.* 2004;19(10):2319–24.
124. Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, et al. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Mol Hum Reprod.* 2010;16(8):557–69.
125. Wale PL, Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod.* 2012;87(1):24, 1–8.
126. Wale PL, Gardner DK. Oxygen affects the ability of mouse blastocysts to regulate ammonium. *Biol Reprod.* 2013;89(3):75.
127. Seli E, Sakkas D, Scott R, Kwok SC, Rosendahl SM, Burns DH. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil Steril.* 2007;88(5):1350–7.
128. Ahlström A, Wikland M, Rogberg L, Barnett JS, Tucker M, Hardarson T. Cross-validation and predictive value of near-infrared spectroscopy algorithms for day-5 blastocyst transfer. *Reprod Biomed Online.* 2011;22(5):477–84.
129. Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: A prospective blinded pilot study. *Fertil Steril.* 2008;90(1):77–83.
130. Seli E, Bruce C, Botros L, Henson M, Roos P, Judge K, et al. Receiver operating characteristic (ROC) analysis of day 5 morphology grading and metabolomic Viability Score on predicting implantation outcome. *J Assist Reprod Genet.* 2011;28(2):137–44.
131. Vergouw CG, Botros LL, Roos P, Lens JW, Schats R, Hompes PG, et al. Metabolomic profiling by near-infrared spectroscopy as a tool to assess embryo viability: A novel, non-invasive method for embryo selection. *Hum Reprod.* 2008;23(7):1499–504.
132. Pais RJ, Sharara F, Zmuidzinaite R, Butler S, Keshavarz S, Iles R. Bioinformatic identification of euploid and aneuploid embryo secretome signatures in IVF culture media based on MALDI-ToF mass spectrometry. *J Assist Reprod Genet.* 2020;37(9):2189–98.
133. Katz-Jaffe MG, Gardner DK, Schoolcraft WB. Proteomic analysis of individual human embryos to identify novel biomarkers of development and viability. *Fertil Steril.* 2006;85(1):101–7.
134. Katz-Jaffe MG, McReynolds S, Gardner DK, Schoolcraft WB. The role of proteomics in defining the human embryonic secretome. *Mol Hum Reprod.* 2009;15(5):271–7.
135. Katz-Jaffe MG, Schoolcraft WB, Gardner DK. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. *Fertil Steril.* 2006;86(3):678–85.
136. Lee YS, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod.* 2015;30(3):543–52.

137. Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev.* 2015;27(4):638–54.
138. Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod.* 2009;80(2):295–301.
139. Venturas M, Shah JS, Yang X, Sanchez TH, Conway W, Sakkas D, et al. Metabolic state of human blastocysts measured by fluorescence lifetime imaging microscopy. *Hum Reprod.* 2022;37(3):411–27.
140. Shah JS, Venturas M, Sanchez TH, Penzias AS, Needleman DJ, Sakkas D. Fluorescence lifetime imaging microscopy (FLIM) detects differences in metabolic signatures between euploid and aneuploid human blastocysts. *Hum Reprod.* 2022;37(3):400–10.
141. Tan TCY, Mahbub SB, Campbell JM, Habibalahi A, Campugan CA, Rose RD, et al. Non-invasive, label-free optical analysis to detect aneuploidy within the inner cell mass of the preimplantation embryo. *Hum Reprod.* 2021;37(1):14–29.
142. Trimarchi JR, Liu L, Porterfield DM, Smith PJ, Keefe DL. A non-invasive method for measuring preimplantation embryo physiology. *Zygote.* 2000;8(1):15–24.
143. Trimarchi JR, Liu L, Smith PJ, Keefe DL. Noninvasive measurement of potassium efflux as an early indicator of cell death in mouse embryos. *Biol Reprod.* 2000;63(3):851–79.
144. Lopes AS, Larsen LH, Ramsing N, Løvendahl P, Räty M, Peippo J, et al. Respiration rates of individual bovine in vitro-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. *Reproduction.* 2005;130(5):669–79.
145. Ottosen LD, Hindkjaer J, Lindenberg S, Ingerslev HJ. Murine pre-embryo oxygen consumption and developmental competence. *J Assist Reprod Genet.* 2007;24(8):359–65.
146. Jurisicova A, Casper RF, MacLusky NJ, Mills GB, Librach CL. HLA-G expression during preimplantation human embryo development. *Proc Natl Acad Sci U S A.* 1996;93(1):161–5.
147. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G, expressed in human trophoblasts. *Science.* 1990;248(4952):220–3.
148. Noci I, Fuzzi B, Rizzo R, Melchiorri L, Criscuoli L, Dabizzi S, et al. Embryonic soluble HLA-G as a marker of developmental potential in embryos. *Hum Reprod.* 2005;20(1):138–46.
149. Sher G, Keskintepe L, Nouriani M, Roussev R, Batzofin J. Expression of sHLA-G in supernatants of individually cultured 46-h embryos: A potentially valuable indicator of 'embryo competency' and IVF outcome. *Reprod Biomed Online.* 2004;9(1):74–8.
150. Yie SM, Balakier H, Motamed G, Librach CL. Secretion of human leukocyte antigen-G by human embryos is associated with a higher in vitro fertilization pregnancy rate. *Fertil Steril.* 2005;83(1):30–6.
151. Ménézo Y, Elder K, Viville S. Soluble HLA-G release by the human embryo: An interesting artefact? *Reprod Biomed Online.* 2006;13(6):763–4.
152. Sageshima N, Shobu T, Awai K, Hashimoto H, Yamashita M, Takeda N, et al. Soluble HLA-G is absent from human embryo cultures: A reassessment of sHLA-G detection methods. *J Reprod Immunol.* 2007;75(1):11–22.
153. Sargent I, Swales A, Ledee N, Kozma N, Tabiasco J, Le Bouteiller P. sHLA-G production by human IVF embryos: Can it be measured reliably? *J Reprod Immunol.* 2007;75(2):128–32.
154. O'Neill C. The role of paf in embryo physiology. *Hum Reprod Update.* 2005;11(3):215–28.
155. Stamatkin CW, Roussev RG, Stout M, Absalon-Medina V, Ramu S, Goodman C et al. PreImplantation Factor (PIF) correlates with early mammalian embryo development-bovine and murine models. *Reprod Biol Endocrinol.* 2011;9(63). doi: [10.1186/1477-7827-9-63](https://doi.org/10.1186/1477-7827-9-63). PMID: 21569635; PMCID: PMC3112407.
156. Strom CM, Bonilla-Guererro R, Zhang K, Doody KJ, Tourgeman D, Alvero R, et al. The sensitivity and specificity of hyperglycosylated hCG (hhCG) levels to reliably diagnose clinical IVF pregnancies at 6 days following embryo transfer. *J Assist Reprod Genet.* 2012;29(7):609–14.
157. Xiao-Yan C, Jie L, Dang J, Tao L, Xin-Ru L, Guang-Lun Z. A highly sensitive electrochemiluminescence immunoassay for detecting human embryonic human chorionic gonadotropin in spent embryo culture media during IVF-ET cycle. *J Assist Reprod Genet.* 2013;30(3):377–82.
158. Dominguez F, Meseguer M, Aparicio-Ruiz B, Piqueras P, Quiñonero A, Simón C. New strategy for diagnosing embryo implantation potential by combining proteomics and time-lapse technologies. *Fertil Steril.* 2015;104(4):908–14.
159. Thapa S, Heo YS. Optimization of a single substrate-based fluorometric assay for glucose and lactate measurement to assess pre-implantation single embryo quality and blood in obese mouse and clinical human samples. *Anal Chem.* 2022;94(46):16171–9.
160. Rubio C, Navarro-Sánchez L, García-Pascual CM, Ocalí O, Cimadomo D, Venier W et al. Multicenter prospective study of concordance between embryonic cell-free DNA and trophectoderm biopsies from 1301 human blastocysts. *Am J Obstet Gynecol.* 2020;223(5):751.e.

EVALUATION OF EMBRYO QUALITY

Time-Lapse Imaging to Assess Embryo Morphokinetics

Akhil Garg, María Ángeles Valera, and Marcos Meseguer

Introduction

In vitro fertilization (IVF) programs are coming closer every day to the goal of reducing multiple pregnancies while maintaining good clinical results. The transfer of a single embryo progressively became a reality specifically in developed countries, and this is the result of major improvements in different areas. From a clinical point of view, two major achievements are worth mentioning: first, physicians have learned to handle the stimulation drugs that are purer, more powerful, and more comfortable for the patient; and, second, an increased knowledge of the pathophysiology of ovarian hyperstimulation syndrome has made the frequency of this syndrome almost anecdotal. On the other hand, concerns about the “epidemic” of multiple gestations have raised awareness of the risks not only to the mother (gestational diabetes, hypertension, and anaemia) but also to the babies—extreme prematurity, low birth weight, children with neurological damage, and so on—not to mention the psychological burden and suffering of the parents and the tremendous health costs that it entails. From the laboratory point of view, several achievements are worth mentioning as well: studies on embryo metabolism have led to the formulation of suitable culture media. In the early 1990s, the introduction of intracytoplasmic sperm injection (ICSI) revolutionized the treatment of male infertility and genetic screening became the gold standard for the selection of aneuploid embryos. Vitrification came along, and preservation of fertility was no longer a utopia for modern women; the wave of the “omics” initiated an era of non-invasiveness for studying human embryos in the laboratory, and most recently in the last decade, the introduction of imaging systems, artificial intelligence (AI), and machine learning allowed us to assess embryos in a different way: through their morphokinetics and analysis of images and video captured during the early embryonic development.

Time-lapse technology

The success of an IVF treatment mainly depends on two factors, (i) optimal incubation environment and (ii) accurate embryo selection apart from other factors such as stimulation, endometrial preparation, etc. According to recent trend, the success of an IVF cycle is measured in terms of live birth instead of implantation. Traditional embryo assessment is based on time point evaluations. Through this approach, embryo categories are normally based on the number of blastomeres and nuclei, the percentage of fragments, cell symmetry, and the quality of the inner cell mass (ICM) and trophectoderm (TE). Even though great knowledge has been achieved through this approach, it has been demonstrated that embryo status can markedly change within a few hours [1–4]. In addition, inter- and intra-observer variabilities are commonly described problems [5], probably due to the subjective nature surrounding traditional morphological assessment [4, 6–10]. In

theory, increasing the number of observations could provide better information on the development of the embryo and therefore improve its assessment [4, 11, 12]. However, increased handling and higher evaluation frequencies will expose the embryo to undesirable changes in temperature, humidity, and gas composition [11–13]; apart from these exposure to light is also harmful to embryos.

Time-lapse technology (TLT) represents a solution to this problem. In 1997, Payne et al. [14] developed time-lapse cinematography to manage intermittent observation of the process of oocyte fertilization. Later, the observation period was augmented while maintaining optimal culture conditions [2], and nowadays TLT allows the complete observation of the entire process of embryo development in the IVF laboratory. The two main advantages of these systems are (i) improved and stable culture conditions and (ii) the determination of objective and accurate markers, both quantitative and qualitative [4]. In addition, we should mention that there is reduced handling and human risk; minimization of culture media, gas, and oil; detection of abnormal events that would normally occur between observations; reduced inter- and intra-observer variability; and reduced numbers of hours needed by the embryologist in the laboratory [15].

In 2011, Meseguer et al. coined the term “morphokinetics,” defined as the combination of the embryo appearance (morphology) and the timing in which cellular events occur. Morphokinetics has been introduced as a new concept to improve embryo selection. The use of this strategy could allow single-embryo transfers (SETs) without jeopardizing the overall IVF success [16], becoming very attractive, especially in European countries in which legislation is stricter about the number of embryos transferred [15].

Theoretically, the uninterrupted culture represents an improvement to the conventional incubator, where embryos are removed from the incubator for inspection at each time point for evaluation. There have been several studies on TLT in the last decade regarding blastocyst formation, blastocyst quality, pregnancy, implantation, live birth, aneuploidy, and other patient characteristics to measure the benefit and usefulness of the system through morphokinetic parameters and other observations which were not possible with conventional morphology and incubators. Apart from that, new knowledge and information has been acquired through this technology regarding early embryo development and the potential of non-invasive markers. Recently promising machine learning (ML) technology, part of AI and computer vision (CV), has overtaken the field in terms of creating selection algorithms.

Models on the market

There are different options of TLT available on the market. Some of them present all the items integrated into one single piece of equipment (e.g. Embryoscope®, Embryoscope+® [Vitrolife], Geri®, Geri+® [Genea Biomedix], Miri® TL [Esco Medical], and ASTEC™).

Others offer the option of introducing a microscope inside an available incubator (e.g. Primo Vision® [Vitrolife] and the Eeva™ Test [Merck-Serono]). Different systems also provide different embryo selection algorithms and the capability to create one's own algorithm or use the algorithm from other algorithms available in the market. Table 17.1 describes the clinical and technical features of all the TLT available on the market [17].

Kinetic parameters (individual plus calculated)

As described in the proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group [18], Table 17.2 describes the morphokinetic "individual" variables.

Additionally, Table 17.3 defines "calculated" variables that represent a certain cell stage or cycle duration, and during the de-vitrification process we can also define the duration of re-expansion and completion of re-expansion (Figure 17.1).

Blastocyst development studies

After the first study by Payne et al., in 1997, on human embryos, TLT first appeared in the market in 2009, and only a few IVF laboratories adopted the system in starting because of cost. But now TLT is widely used in IVF laboratories. Several studies have been conducted since 2008 to associate the morphokinetics markers with good-quality embryos and the prediction of blastocyst formation (Table 17.4).

After TLT appeared in the market, the potential of technology shifted from mere observation of human embryos while in culture to a selection and prediction tool. With TLT, a substantial amount of novel information about embryo development is acquired. The challenge has been how to use this information. Many studies have tried to assess the embryo based on different potential kinetic markers, such as time interval between cell division, fertilization markers, cleavage features (e.g. direct cleavage, absent cleavage, chaotic cleavage, reverse cleavage, etc.), multinucleation, compaction, symmetry, fusion, fragmentation, collapse, expansion, uneven blastomere, hatching, etc., to determine the highest chances for achieving success. Most of the studies in the past have been done on EmbryoScope™; and a few studies on time-lapse incubators which can be integrated in conventional incubators, such as EEVA™ and PrimoVision™, as other time-lapse incubators like Geri™, Miri™, etc., were introduced later in the market.

In 2010, Wong et al. analysed kinetic parameters of 100 embryos that were cultured up to days 5 or 6 of development [19] and found out three predictors of blastocyst formation: P1—duration of the first cytokinesis (14.3 ± 6.0 minutes); P2—interval between the end of the first mitosis and the initiation of the second mitosis (11.1 ± 2.2 hours); and P3—the synchrony between the second and third mitosis (1.0 ± 1.6 hours). The authors concluded that embryo development to the blastocyst stage could be predicted with 94% sensitivity and 93% specificity after using parameters P1, P2, and P3. Embryos with one or more values outside these ranges were expected to arrest. The time of completion of the second and third mitosis was also analysed by Hashimoto et al., who observed that high-scoring blastocysts took significantly shorter times for these divisions [20]. Later Coticchio et al. observed faster fertilization kinetics was associated with better embryo development, and new kinetic parameters were defined: cytoplasmic halo appearance (tHaloA) → disappearance (tHaloD); halo appearance (tHaloD) → PN fading (tPNf); PN fading (tPNf) → first cleavage

(t2); and male PN appearance (tPNa_m) → male PN fading (tPNf_m) [21]. After the study from Wong et al., a lot of studies tried to examine different morphokinetic parameters linked to blastocyst formation and blastocyst quality based on selection and deselection model. A few algorithms have also been proposed by different groups, most of the algorithms proposed earlier were based on a hierarchical model.

In a retrospective cohort study, Cruz et al. [22] monitored 834 embryos and defined kinetic markers linked to embryo development (Figure 17.2). In the same year, Dal Canto et al. [23] observed different cleavage kinetic parameters linked to embryo development and found that early cleavage from 2- to 8-cell stage occurs progressively earlier in embryos with the ability to develop to blastocyst and expand.

In 2013, Chamayou et al. [24] reported time intervals of morphokinetic parameters identified as predictors of embryo competence. The authors concluded that day-3 embryos developed into viable blastocysts when their kinetic parameters met the following ranges: t1 (18.4–30.9 hpi [hours post insemination]), t2 (21.4–34.8 hpi), t4 (33.1–57.2 hpi), t7 (46.1–82.5 hpi), t8 (46.4–97.8 hpi), tC – tF (7.7–22.9 hpi), and s3 (0.7–30.8 hpi). In the same year, a study by Kahraman et al. found a statistically significant difference in kinetics parameters t2, t8, tM, tB, cc2, s2 between top-quality blastocyst and good-quality blastocyst versus poor-quality blastocyst [25].

A couple of prospective studies were performed in 2013. Kirkegaard et al. [28] analysed 571 embryos from good prognosis patients and reported three markers linked to high-quality blastocysts: duration of the first cytokinesis, duration of the 3-cell stage, and direct cleavage to the 3 cell, all of which had comparable predictive values but no connection to implantation results. The Wong et al. study was the origin of EEVA™ test, and later Conaghan et al. [29] conducted a two-phase multicentre study to develop and validate an algorithm to predict blastocyst formation. A total of 1727 embryos were monitored by automatic cell-tracking software. The time between cytokinesis 1 and 2 (P2) and the time between cytokinesis 2 and 3 (P3) turned out to be the strongest parameters in the prediction model. The results indicated a higher probability of usable blastocyst formation when both P2 and P3 were within specific cell division timing ranges (P2, 9.33–11.45 hours; P3, 0–1.73 hours) and a lower probability when either P2 or P3 were outside the specific cell timing ranges.

The Conaghan model, also known as EEVA I, was tested retrospectively by a different group using a set of 1519 transferred embryos with known clinical outcome [31]. According to the algorithm, embryos were classified as usable or non-useable based on EEVA high and EEVA low score. The difference in implantation rate between the usable group and the whole cohort was 30%, indicating that implantation rates could increase using this model. In addition, the percentage of non-useable embryos that resulted in implantation was 50.6%, raising concerns regarding the discarding of viable embryos. Even though the Conaghan model was developed for blastocyst formation and the end point of this study was clinical outcome, the authors expressed that an implanted embryo should derive from the usable embryo group and not from the non-useable group (or at least not in such high proportions). The possible explanation for these findings, according to the authors, could be that the model is based on narrow time intervals (Figure 17.3).

In 2015, Cetinkaya et al. [33] studied 17 kinetic markers in 3354 embryos cultured up to day 5. The parameters t8 – t5, cleavage

TABLE 17.1 Technical and Clinical Features Compared between the Time-Lapse Systems Available on the Market

Feature	System A	System B	System C	System D	System E	System F	System G
System	ASTEC™	Primo Vision™	ESD+®	ESD®	MIRI®	Geri®	Geri+®
Incubator	Integrated	Placed in conventional incubators	Integrated	Integrated	Integrated	Integrated	Integrated
External dimensions (W × D × H mm)	382 × 590 × 219	220 × 80 × 110	550 × 600 × 500	603 × 560 × 435	805 × 585 × 375; 950 × 685 × 375	615 × 500 × 300	615 × 500 × 300
Specific culture dish	Single culture	Group culture	Group culture (shared medium)	Single culture	Single or group culture (shared medium)	Group culture (shared medium)	Group culture (shared medium)
Specification							
Number of focal planes	11 (max.)	3 to 11	11	Up to 17; typically 7	3 to 7	Up to 11	Up to 11
Time between acquisitions	15 min. (adjustable between 15 and 60 min.)	5 to 60 min.	10 min.	10 min. for 7 focal planes, 2 min. for a single focal plane	5 min.	5 min.	5 min.
Camera (megapixels)	1.3	5 (1 px/μm)	2.2 (3 px/μm)	1.3 (3 px/μm)	1.25	5	5
Type of microscopy	Oblique illumination	Brightfield (Hoffman modulation)	Brightfield (Hoffman modulation)	Brightfield (Hoffman modulation)	Brightfield	Brightfield	Brightfield/darkfield
Embryo illumination for image	Red LED (623 nm)	Adjustable green LED (550 nm)	Red LED (630 nm)	Red LED (635 nm)	Red LED (635 nm)	Orange LED (591 nm)	Red LED (630 nm)
Time of light exposure	0.008 s	0.2 to 0.005 s	<0.02 s	<0.032 s	0.064 s	<0.005 s	<0.005 s; <0.009 s
Software							
Morphokinetics annotation	Yes, manual	Yes, manual, guided/semi-automated	Yes, manual, guided/semi-automated/fully automated	Yes, manual, guided/semi-automated	Yes, manual and automated	Yes, manual, semi-automated and automated	Yes, manual, semi-automated and automated
Predictive algorithm	/	Yes, or defined by user	Yes, or defined by user	Yes, or defined by user	Defined by user	Defined by user	Yes
Gaseous Condition							
Gas consumption	N/A	N ₂ ; max 5L/h, Typical 2–3 L/h, CO ₂ ; max 2 L/h, Typical 0.5 L/h	N ₂ ; <10 L/h, Typical 3 L/h, CO ₂ ; <1 L/h,	N ₂ ; 3–5 L/h, CO ₂ ; 1–2 L/h,	N ₂ & CO ₂ ; 3.6 L/h	N ₂ & CO ₂ ; 3.6 L/h	
Type of gas	Built-in gas mixer	N/A	Integrated gas mixer	Integrated gas mixer	Built-in gas mixer; premixed not required	Premixed	Premixed
Recovery time (min)	Temperature: 10–12 Gas: 5–6	N/A	CO ₂ <5; O ₂ <3	CO ₂ and temperature <5; O ₂ <15	Temperature <1; gas <3	Temperature <1; CO ₂ <3; humidity 4 h (for full recovery)	Temperature <1; CO ₂ <3; humidity 4 h (for full recovery)

(Continued)

TABLE 17.1 Technical and Clinical Features Compared between the Time-Lapse Systems Available on the Market (Continued)

Feature	System A	System B	System C	System D	System E	System F	System G
Other							
Dry or humid culture system	Dry	N/A	Dry	Dry	Dry	Dry or humid, independently on each chamber	Dry or humid, independently on each chamber
pH monitoring	Possible	N/A	Specific pH validation dish	Possible	Built-in	Possible	Possible
Capacity	12 embryos/dish 9 dishes/ incubator	16 or 9 embryos/dish 1 dish/ inverted microscope	16 embryos/ dish 15 dishes/ incubator	12 embryos/dish 6 dishes/ incubator	14 embryos/dish 6 or 12 dishes/ incubator	16 embryos/dish 6 dishes/ incubator	16 embryos/dish 6 dishes/incubator
Electronic record system	Manually	Possible to integrate	Possible to integrate	Possible to integrate	Under development	Possible to integrate	Possible to integrate
Remote access to image	Yes	Yes	Yes	Yes	Yes	Yes	Yes

TABLE 17.2 Morphokinetics Individual Variables

t0	Time of IVF or mid-time of microinjection (ICSI/intracytoplasmic morphologically selected sperm injection)
tPB2	The second polar body completely detached from the oolemma
tPN	Fertilization is confirmed
tPNa	The appearance of individual pronuclei: tPN1a, tPN2a, tPN3a, etc.
tPNf	Time of pronuclei disappearance: tPN1f, tPN2f, etc.
tZ	Time of PN scoring
t2 to t9	Time to two to nine discrete cells
tTM	Trichotomous mitosis at different stages
tSC	The first evidence of compaction
tMf/p	End of compaction "f" corresponds to full compaction "p" corresponds to partial compaction
tSB	Initiation of blastulation
tByz	Full blastocyst "y" corresponds to morphology of ICM "z" corresponds to morphology of TE cells
tEyz	Initiation of expansion; first frame of zona thinning
tHNyz	Herniation; end of expansion phase and initiation of hatching
tHDyz	Fully hatched blastocyst

synchronicity from four to eight cells (CS4–8), and cleavage synchronicity from two to eight cells (CS2–8) were found to be good indicators. In particular, CS2–8, defined as $([t_3 - t_2] + [t_5 - t_4]) / (t_8 - t_2)$, was selected as the best predictor on day 3 for blastocyst formation and quality (area under the curve [AUC] = 0.786).

Yang et al. [34] took a different approach and developed a study to describe different types of abnormal divisions and how they may affect the developmental potential of the embryo. Seven types of divisions within two categories were defined according to the impact caused on blastocyst development. Category

TABLE 17.3 Morphokinetics Calculated Variables

VP	$t_{PNf} - t_{PNa}$	Pronucleus (PN) duration
ECC1	$t_2 - t_{PB2}$	Duration of first cell cycle
ECC2	$t_4 - t_2$	Duration of second cell cycle
cc2		Duration of single blastomere second cell cycle: $cc2a = t_3 - t_2$; $cc2b = t_4 - t_2$
cc3		Duration of single blastomere third cell cycle: $cc3a = t_5 - t_4$; $cc3b = t_6 - t_4$; $cc3c = t_7 - t_4$; $cc3d = t_8 - t_4$
ECC3	$t_8 - t_4$	Duration of third cell cycle
s2	$t_4 - t_3$	Synchronization of cell divisions
s3	$t_8 - t_5$	Synchronization of cleavage pattern
Dcom		Duration of compaction $t_{Mf} - t_{SC}$ (full compaction); $t_{Mp} - t_{SC}$ (partial compaction)
dB	$t_B - t_{SB}$	Duration of blastulation
Dexp	$t_{HN} - t_E$	Duration of blastocyst expansion
Dcol	$t_{BCend(n)} - t_{BCi(n)}$	Duration of blastocyst collapse "n" is number of episodes of collapse and re-expansion
dre – exp		Duration of re-expansion
tre – exp		
end(n) – tre – expi(n)		
dHN	$t_{HN} - t_{HD}$	Duration of herniation
tRE		Time of the start of re-expansion
tCRE		Time of completion of re-expansion

1(minor abnormality) consisted of divisions with low impact on the development potential: normal division, uneven blastomere formation, and appearance of big fragments. Category 2 (major abnormality) consisted of divisions with high impact on embryo development: direct cleavage, fragmentation, developmental arrest, and disordered division. By taking this into consideration,

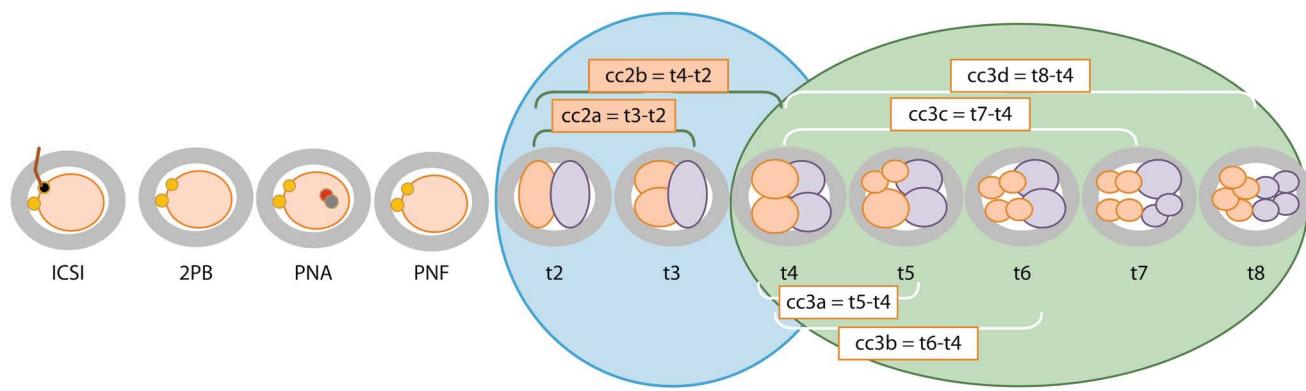


FIGURE 17.1 Graphical representation of kinetic variables up to the 8-cell stage. Abbreviations: ICSI, intracytoplasmic sperm injection; 2PB, two polar bodies; PNa, pronucleus appearance; PNf, pronucleus fading.

TABLE 17.4 Studies Associating Blastocyst Formation with Kinetic Markers

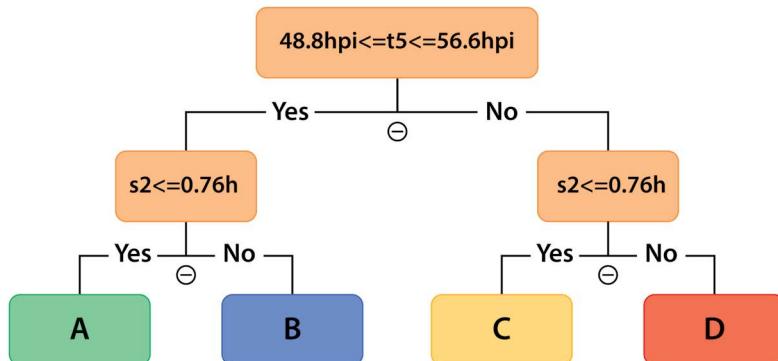
Author	Study Design	Embryos (n)	Embryo Origin	Time-Lapse System	Predictive Marker Identified
Lemmen et al. [26]	Retrospective study	102	IVF/ICSI cycles	Nikon Diaphot 300 microscope with camera in a closed system	T2, PN breakdown
Wong et al. [19]	Retrospective study	100	Supernumerary frozen 2PN	Modified Olympus IX-70/71; CKX-40/41	First cytokinesis, P2 and P3
Hashimoto et al. [20]	Experimental study	80	Donated human embryos for research	Biostation CT	Durations of second (t4 – t3) and third mitotic divisions (t8 – t5)
Hlinka et al. [27]	Retrospective study	180	Clinical IVF routine	Primo vision	c2, c3, and c4; i2, i3, and i4
Cruz et al. [22]	Retrospective cohort study	834	Oocyte donation cycles	EmbryoScope	t4, t5, s2, DC3 cells, and tM; UN2 cells
Dal Canto et al. [23]	Retrospective cohort study	459	Clinical IVF routine	EmbryoScope	t3, t4, t5, t6, t7, t8, t3 – t2, t4 – t3, t4 – t2, t8 – t4, t8 – t5
Kahraman et al. 2013 [25]	Randomized controlled trial	406	Clinical IVF routine	Embryoscope	cc2, s2, t2, t8, tM, tB
Chamayou et al. [24]	Retrospective study	224	Fresh oocyte ICSI treatments	EmbryoScope	t1, t2, t4, t7, t8, tC – tF, and s3
Kirkegaard et al. [28]	Prospective cohort study	571	Fresh oocyte ICSI treatments	EmbryoScope	First cytokinesis, t3, and DC3 cells
Conaghan et al. [29]	Prospective multicentre cohort study	1233	Fresh oocyte ICSI treatments	Eeva	P2 and P3
Wirkka et al. [30]	Retrospective multicentre cohort study	651	Clinical IVF routine	Eeva	AS, A1 ^{cyt} , CC, AC
Kirkegaard et al. [31]	Retrospective multicentre study	1519	Fresh oocyte ICSI treatments	EmbryoScope	t3 – t2, t4 – t3
Desai et al. [32]	Retrospective study	648	ICSI cycles	EmbryoScope	tPNf, t2, t4, t8, t9+, tM, tSB, tB, tEB, cc2, s1, s2, s3, t5 – t4, t5 – t2
Cetinkaya et al. [33]	Retrospective observational cohort study	3354	Clinical IVF routine	EmbryoScope	CS2
Yang et al. [34]	Prospective observational study	345	Metaphase I donated for research	Primo vision	Cleavage patterns
Milewski et al. [35]	Retrospective observational study	432	Fresh oocyte ICSI treatments	EmbryoScope	t2, t5, cc2, and SC

(Continued)

TABLE 17.4 Studies Associating Blastocyst Formation with Kinetic Markers (Continued)

Author	Study Design	Embryos (n)	Embryo Origin	Time-Lapse System	Predictive Marker Identified
Storr et al. [36]	Prospective cohort study	380	Fresh oocyte ICSI treatments	Embryoscope	s3, t8, and tEB
Motato et al. [37]	Retrospective study	7483	Clinical IVF routine	EmbryoScope	tM; t8 – t5
Mizobe et al. [38]	Cohort Study	791	ICSI/IVF	EmbryoScope	Fragmentation based
Liu et al. 2016 [39]	Retrospective cohort study	270	ICSI	EmbryoScope	s2, t5 – tPNf
Zhan et al. 2016 [40]	Retrospective observational study	21261	ICSI/IVF cycles	Embryoscope	DUC
Mizobe et al 2018 [41]	Cohort Study	948	ICSI treatment	EmbryoScope	EC, HS
Coticchio et al. 2018 [21]	Retrospective cohort study	500	ICSI treatment	EmbryoScope	t2 – tPNf, tPN _m – tPNf _m , tPNf – tHaloA, tHaloD – tHaloA
Fishel et al. 2018 [42]	Retrospective cohort study	843	ICSI/IVF treatment	Embryoscope	tSB, dB
Desai et al. 2018 [43]	Retrospective study	1478	ICSI cycles	Embryoscope	IDC, DUC, 2+ dysmorphism (MN, RC, IDC, DUC), tSB, tEB, tEB – tSB
Lagalla et al. 2020 [44]	Retrospective study	499	ICSI cycles	Embryoscope	FCM
Pennetta et al. 2021 [45]	Retrospective cohort study	780	ICSI/IVF treatment	Embryoscope	s2

Abbreviations: ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; HS, high synchrony; FCM, full compaction; DUC, direct uneven cleavage; IDC, irregular chaotic division.

**FIGURE 17.2** Blastocyst development algorithm (described by [22]). Abbreviation: hpi, hours post insemination.**FIGURE 17.3** Original embryo categorization algorithm known as EEVA I. (Based on data from [29]).

a hierarchical classification model was developed based on the division patterns during the three initial embryo cleavages rather than on morphokinetic parameters as in previous studies. Day-3 embryos were then classified into six categories of A–F according to the number and category of the abnormal cleavages they had presented (Figure 17.4).

In a study by Milewski et al., the parameters t2, t3, t4, t5, cc2, and s2 were measured and differences were observed between embryos that reached the blastocyst stage and embryos that arrested. A total of 432 embryos were analysed. The resultant data for each parameter were divided into four intervals (C1–C4),

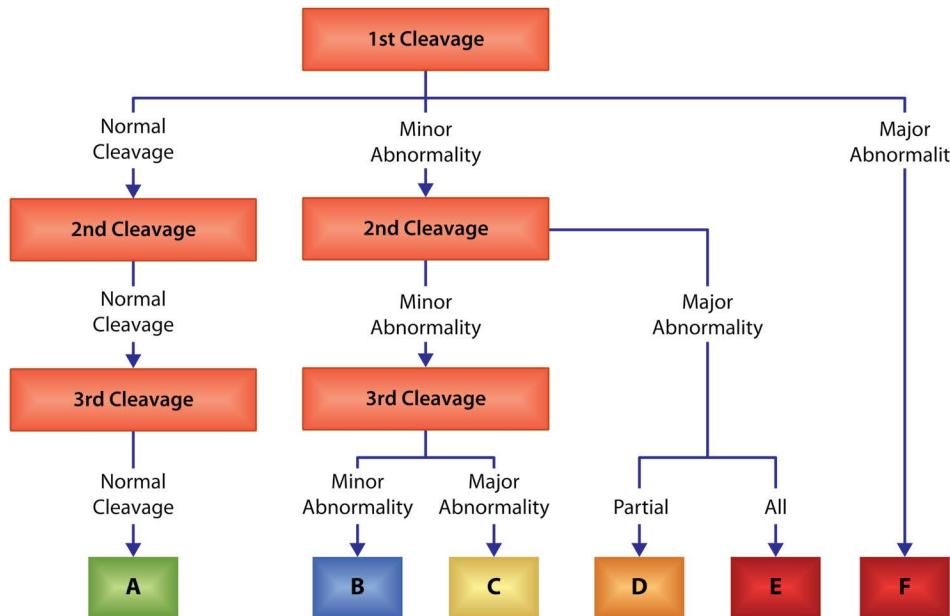


FIGURE 17.4 Yang algorithm for blastocyst development based on cleavage feature. (Based on data from [34].)

and score values were assigned in order to find out which parameter values corresponded to the highest blastocyst development rate. The highest ones generally belonged to compartments C3 and C2. The extreme compartments—C1 and C4—had the lowest rates. A univariate logistic regression analysis concluded that all the studied parameters were significantly associated with blastocyst development. However, after multivariate logistic regression, only the t₂, t₅, and cc₂ parameters were considered and combined into a new parameter (SC), defined as the predictor of development to blastocyst [35].

Storr et al. recorded the timings of 380 blastocysts and found eight significant prediction markers of top-quality blastocysts: s₃, t₆, t₇, t₈, tM, tSB, tB, and tEB. Out of these potential predictors, s₃ was identified as the one with the best individual discriminatory capacity before compaction (AUC = 0.585, 95% CI = 0.534–0.635), and tEB was identified as the best predictor regardless of embryo stage (AUC = 0.727, 95% CI = 0.675–0.775). By combining ts₃, tEB, and t₈, a model with higher discriminatory capacity for predicting top-quality embryos was proposed [36].

Motato et al., in 2016 [37], conducted a three-phase observational, retrospective, single-centre clinical study in which the authors describe the events associated with blastocyst formation and implantation based on the largest sample size ever described with time-lapse monitoring.

Phase 1 consisted of embryo scoring based on a classification tree to select embryos with higher blastocyst formation probabilities. The observed correlations between morphokinetic parameters and blastocyst formation were the basis for a proposed hierarchical classification procedure to select viable embryos with a high blastocyst formation potential. A detailed retrospective analysis of cleavage times was made for 7483 zygotes. A total of 17 parameters were studied and several were significantly correlated with blastocyst formation and implantation. The most predictive parameters for blastocyst formation were time of morula formation, tM (81.28–96.0 hours after ICSI), and t₈ – t₅ (\leq 8.78 hours) or time of transition of five-blastomere embryos to eight-blastomere embryos (Figure 17.5).

Phase 2 focused on the blastocysts transferred and implantation rate. Owing to a lack of a relationship between the

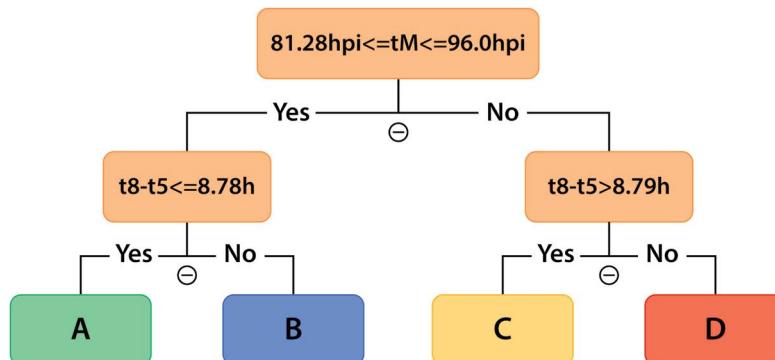
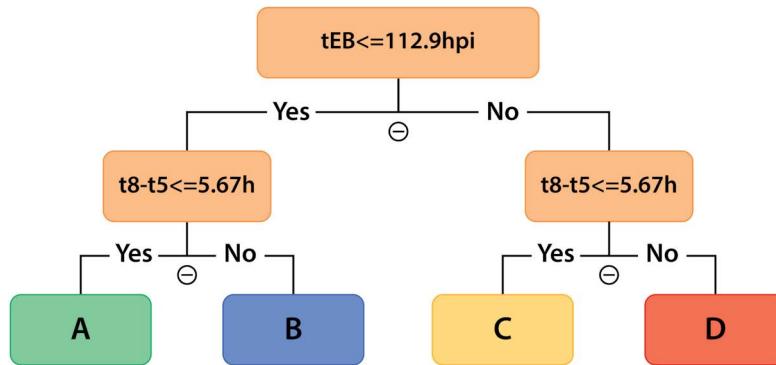


FIGURE 17.5 Algorithm for blastocyst development algorithm described by Motato et al. 2016.

**FIGURE 17.6** Implantation algorithm described by Motato et al. 2016.

previously described variables and implantation potential, the authors identified new variables by comparing transferred blastocysts ($n = 383$) that implanted with those that did not implant ($n = 449$). Once again, they analysed 17 morphokinetic parameters and identified the variables' time for expansion blastocyst, tEB (107.9–112.9 hours after ICSI), and t8 – t5 (5.67 hours after ICSI) as predicting blastocyst implantation, with a ROC value of 0.591 (95% CI = 0.552–0.630). Using these data, a hierarchical model representing a classification tree was proposed. The model subdivided blastocysts into four categories from A to D with higher or lower implantation rates (i.e. from 72.2% in category A to 39.7% in category D) (Figure 17.6). Phase 3 was the validation phase.

The authors concluded that the inclusion of kinetic parameters into score evaluations could improve blastocyst selection criteria and predict blastocyst formation with high accuracy. In addition, the proposed models classify embryos according to their probabilities of blastocyst stage and implantation. In 2018, Fishel et al. observed tSB and dB (tB – tSB) are linked to top-quality blastocyst formation [42]. A few other studies

were performed by Mizobe et al. and observed t2, t4, and high synchrony are linked to blastocyst and top-quality blastocyst formation [38, 41]. In a recent study by Pennetta et al., s2 was linked to blastocyst development [45]. There were a few other studies that didn't find the significant differences in between the conventional incubator and TLT system in terms of blastocyst development [46, 47].

Implantation, pregnancy, and live-birth studies

In addition to blastocyst formation, the scientific community has also correlated kinetic markers to embryo implantation and live birth as an end point which is a main factor for a successful IVF cycle (Table 17.5).

Starting in 2008, Lemmen et al. [26] retrospectively compared time-lapse recordings of a small group of embryos transferred at the 4-cell stage that resulted in eight pregnancies. In this case, the authors observed that nuclei appearance in the first blastomere

TABLE 17.5 Implantation and Live Birth Studies

	Study Design	Total Number of Embryos	Embryo Origin	Time-Lapse System	Predictive Marker Identified/Utilized
Lemmen et al. (2008) [26]	Retrospective study	19	IVF/ICSI cycles	Nikon Diaphot 300 microscope with camera in a closed system	Nuclei appearance in the first blastomere
Meseguer et al. (2011) [1]	Retrospective study	247	ICSI cycles	EmbryoScope	t5, s2, cc2, UN 2 cell, MN 4 cell, DC 1–3 cells
Arazello et al. (2012) [50]	Prospective study	159	ICSI cycles	EmbryoScope	PN breakdown
Hlinka et al. (2012) [27]	Retrospective study	114	ICSI cycles	Primovision	c2, c3, and c4; i2, i3, and i4
Rubio et al. (2012) [51]	Multicentre retrospective study	5225 (1659 transferred)	IVF cycles from donated and autologous oocytes	EmbryoScope	DC 2–3 cells
Dal canto et al. (2012) [23]	Retrospective study	134	ICSI/IVF cycle	EmbryoScope	t8
Cruz et al. 2012 [22]	Retrospective study	120	ICSI Donor Oocyte	EmbryoScope	t5, s2
Freour et al. (2013) [52]	Retrospective analysis and prospectively collected database	191	ICSI cycles	EmbryoScope	t4 and s3

(Continued)

TABLE 17.5 Implantation and Live Birth Studies (Continued)

	Study Design	Total Number of Embryos	Embryo Origin	Time-Lapse System	Predictive Marker Identified/Utilized
Chamayou et al. (2013) [24]	Retrospective study	178	ICSI cycles	EmbryoScope	cc3
Kirkegaard et al. (2013) [28]	Prospective cohort study	84	ICSI cycles	EmbryoScope	None
Desai et al. 2014 [32]	Retrospective Study	105	ICSI cycles	EmbryoScope	tPNf, t2, t3, t5, t8, s1, and t5 – t2
Rubio et al. (2014) [48]	Prospective randomized control trial	775	ICSI cycles from donated oocytes	EmbryoScope	T5; s2; cc2; UN 2 cell; MN 4 cell; DC 1–3 cells
Aguilar et al. (2014) [53]	Retrospective cohort study	1448	ICSI cycles from donated oocytes	EmbryoScope	Time to 2PB; PF; length of S-phase
Basile et al. (2015) [54]	Retrospective multicentric study	1122	ICSI cycles from donated and autologous oocytes	EmbryoScope	cc2, t3, t5, UN 2 cell, MN 4 cell, DC 1–3 cells
Ergin et al. (2014) [55]	Retrospective Study	686	ICSI/IVF cycles	Embryoscope	MN
Vermileya et al. (2014) [56]	Retrospective multicentric study	331	IVF/ICSI cycles	EEVA	P2 and P3
Freour et al. (2015) [57]	Retrospective study	528	ICSI cycles	EmbryoScope	t5, s2, cc2, UN 2 cell, MN 4 cell, DC 1–3 cells
Siristatidis et al. (2015) [49]	Prospective Cohort study	239	ICSI cycles	PrimoVision	t2, cc2a, t3, s2, t4, cc3a, t5, s3, t8
Marcos et al. (2015) [58]	Retrospective cohort study	715	ICSI cycles	Embryoscope	Blastocyst contraction
Dominguez et al. (2015) [59]	Retrospective cohort study	28	ICSI cycles from donated oocytes	EmbryoScope	Cc2
Liu et al. (2016) [39]	Retrospective cohort study and prospective validation	336 (270 for study and 66 for validation)	IVF/ICSI cycles	Embryoscope	s2, t5 – tPNf, cells 68 hrs <8, abnormal cleavage
Adamson et al. (2016) [60]	Prospective concurrent cohort study		ICSI and IVF cycles from autologous oocytes	EEVA	P2 and P3
Wu. et al. [61]	Retrospective study	212	Clinical IVF routine	Primo Vision	tPNf, t2, t4, t4 – t3
Goodman et al. (2016) [62]	Prospective randomized control trial	2092	ICSI and IVF cycles from autologous oocytes	Embryoscope	Cc2, s2, t5, s3, tSB, MN, irregular division
Petersen et al. (2016) [63]	Retrospective evaluation (multicentre)	3275	ICSI and IVF cycles	Embryoscope	t3 – tPNf, (t5 – t3/t5 – t2), cells 66 hrs <8
Zhan et al. (2016) [40]	Retrospective observational study	3189	ICSI/IVF cycles	Embryoscope	DUC
Desai et al. (2016) [64]	Retrospective study	669	Vitrified-warmed blastocyst	Embryoscope	MN, delayed blastulation
Carrasco et al. (2017) [65]	Retrospective study	800	ICSI Cycles	EmbryoScope	t4, t7, s3
Mizobe et al. (2017) [66]	Retrospective study	299	IVF/ICSI cycles	Embryoscope	t2, t4, tC, tB
Ebner et al. (2017) [67]	Retrospective study	144	Devitrification cycle	Miri	tRE, tCRE, tCRE–tRE
Kovacic et al. (2018) [68]	Retrospective observational study	143	Devitrification cycle	PrimoVision	tCRE–tRE
Gonzalez et al. (2018) [69]	Retrospective study	234	Devitrification cycle	EmbryoScope	Blastocyst contraction
Bartolacci et al. (2021) [70]	Retrospective study	1801	ICSI cycle	Embryoscope	tPNf, tPNa, t2, t3, t4, t8, t2–tPNf

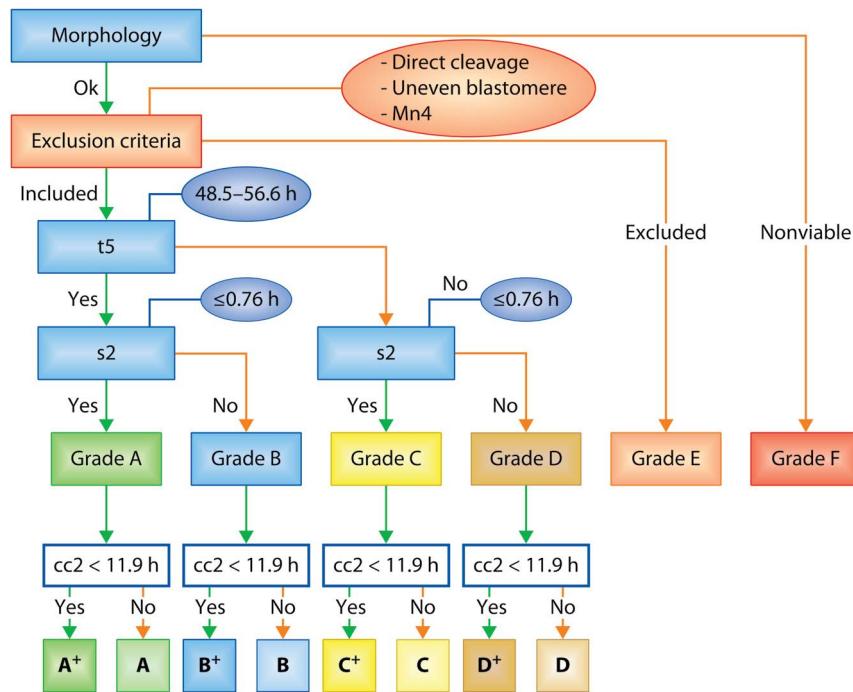


FIGURE 17.7 Original embryo categorization algorithm. (From [1], reproduced with permission.)

following the first cleavage were faster in embryos that implanted versus those that did not, and that nuclei appearance in the first two blastomeres was significantly more synchronous ($p < 0.05$).

Three years later, Meseguer et al. [1] published a study where several parameters were correlated with embryo implantation and suggested a hierarchical algorithm known as the “Meseguer” algorithm or “Meseguer” model. The study was based on 247 known implantation data (KID) embryos that subdivided embryos into six categories from A to F. Four of these categories (A–D) were further subdivided into two subcategories: (+) or (−) (Figure 17.7). The Meseguer model was extensively studied and validated externally by a few studies [25, 48, 49].

In 2012, Azzarello et al. [50] performed a prospective study transferring 159 embryos and proposed the variable “time of pronuclear breakdown” as a predictor of pregnancy. In this study, the pronuclear breakdown of embryos resulting in live births occurred significantly later than those that did not. In fact, the authors proposed the limit of 20 hours and 45 minutes and recommended to avoid transferring embryos presenting pronuclear breakdowns at earlier times.

In the same year, Hlinka et al. [27] proposed a novel method to predict implantation. The model relied on cleavage ratings of the embryos; more specifically, time patterning of cleavage clusters and interphases were used to select the highest-quality embryos. The diagnostic relation between blastocyst implantation and cleavage success was 100% specific for all the embryos analysed ($n = 180$) and all the pregnancies resulted from timely cleaved embryos.

Direct cleavage is another parameter that has been correlated with implantation. Meseguer et al. [1] initially observed this phenomenon based on 247 KID embryos. Later, these findings were confirmed by a multicentre retrospective study performed by Rubio et al. [51]. In this case, the number of embryos

analysed was much higher ($n = 5225$) and embryo implantation for embryos presenting direct cleavage from two to three cells (DC2–3 < 5 hours) was statistically lower than for those with a normal cleavage pattern. Only 1 out of 109 embryos with DC2–3 resulted in clinical pregnancy.

The impact of extrinsic factors on embryo kinetics and their relationship with implantation has been studied as well. In 2013, Freour et al. [52] focused on women who smoked, and the authors observed that embryo divisions occurred later in smokers than in non-smokers, resulting in worse outcomes for the first group. The authors analysed 191 embryos and indicated t4 and s3 as the most relevant kinetic parameters with respect to implantation. According to the distributions of these two variables, implantation was significantly higher in the first two quartiles. Embryos were graded as A or B depending on the optimal range defined for t4 (A = inside the range and B = outside the range). In addition, embryos were given a “+” or “−” value according to the optimal range of s3 (“+” = inside the range and “−” = outside the range). The authors validated this classification model in a database including all transferred embryos, observing implantation rates of 38.7%, 33.3%, 30.7%, and 15.3% for A+, A−, B+, and B− categories, respectively. The proportions of A+ and A− embryos were higher in non-smoker patients.

Chamayou et al. [24] retrospectively compared morphokinetic parameters of 72 implanted and 106 nonimplanted embryos. No differences were found for PN appearance, PN disappearance, t1, t2, t4, t7, t8, tC–tF, and s3 parameters. The authors concluded that these markers were not predictors of implantation, but that they could predict embryo development to the blastocyst stage. In this study, the only predictor marker of implantation and production of a viable pregnancy was cc3.

As opposed to many authors, Kirkegaard et al. [71] showed no differences in the timings of cellular division or embryonic

stage between implanted and non-implanted embryos. The study was based on the observation of 84 SETs. The author identified the duration of first cytokinesis, duration of the 3-cell stage, and direct cleavage to three cells as predictors of high-quality embryo development but not of implantation or pregnancy. Therefore, this group concluded that a universal algorithm for optimal timing might not be feasible.

In 2014, Aguilar et al. [53] studied the human's first cell cycle and its impact on implantation based on morphokinetics. To this aim, the authors conducted a retrospective analysis of 1448 transferred embryos and compared the timings of second polar extrusion, first and second pronuclear appearance, pronuclear abuttal, pronuclear fading, and length of S-phase between implanted and nonimplanted embryos. The time ranges successfully linked to implantation were 3.3–10.6 hours for second polar body extrusion, 22.2–25.9 hours for pronuclear fading, and 5.7–13.8 hours for the length of S-phase.

In 2015, Basile et al. [54] continued the study by Meseguer et al. [1] and published an improved version of the algorithm by studying a larger data set of embryos from four different IVF clinics and included new kinetic parameters (Figure 17.8).

VerMileya et al. [56] extended the EEVA I algorithm to EEVA II and established the relationship between implantation and three embryo categories derived from a computer-automated TLT. The system classified embryos into the categories high EEVA, medium EEVA, or low EEVA based on the variables P2 and P3. According to this multicentre study (205 patients), implantation rates were significantly linked to the three categories; more specifically: 37%, 35%, and 15% for high, medium, and low, respectively. In addition, the clinical pregnancy rate for patients that had one or more "high" transferred embryos was significantly higher (51% vs. 34%; $p = 0.02$) (Figure 17.9). The EEVA algorithm was validated externally by many studies.

Adamson et al., in 2016 [60], tested the same technology in a prospective way. The aim of the study was to prove if an automatic

time-lapse test (TL test) combined with traditional morphology improves day-3 implantation rates compared with morphology alone. Two concurrently collected groups of patients were compared: those who received a day-3 transfer with the use of the TL test (EEVA test) together with morphology (test group), and those who received a day-3 transfer with the use of morphology alone (control group). Analysis of the study's primary end point—implantation rate—showed a significantly higher implantation rate for day-3 transfer among the test group (30.2%, 58/192) than the control group (19.0%, 84/442; $p = 0.003$).

In the same year, Goodman et al. [62] published a new scoring algorithm based on kinetic event t5, s2, s3, tSB, and cc2. The presence of multi-nucleation and irregular division was also included as deselection criteria in the algorithm. The algorithm provides a score between -2 and 4 (Figure 17.10).

Liu et al. [39] validated the "Meseguer" algorithm and derived a new hierarchical model with high AUC in 2016, and this algorithm is known as the "Liu" algorithm, which is based on kinetic parameter s2, t5, and tPNf. Abnormal cleavage and cells at 68 hours less than 8 were described as deselection criteria (Figure 17.11).

Later in the year 2016, Petersen et al. [63] provide a hierarchical algorithm based on KID known as KIDSscore D3 algorithm, which is based on t3, tPNf, t2, and t5. This algorithm also considers the counts of cells should be greater or equal to 8 at 66 hpi (Figure 17.12).

Limitations on the external performance and the universal use of published algorithms have been addressed [11]. In a study published by Freour et al. [52], an external validation of Meseguer et al.'s algorithm [1] was performed in an unselected patient population. The model was applied showing a heterogeneous distribution of implantation rates in the resultant categories. In addition, correlation coefficients were significantly lower than the ones in the original study. However, a simplified version of the model (in which only the two main morphokinetic

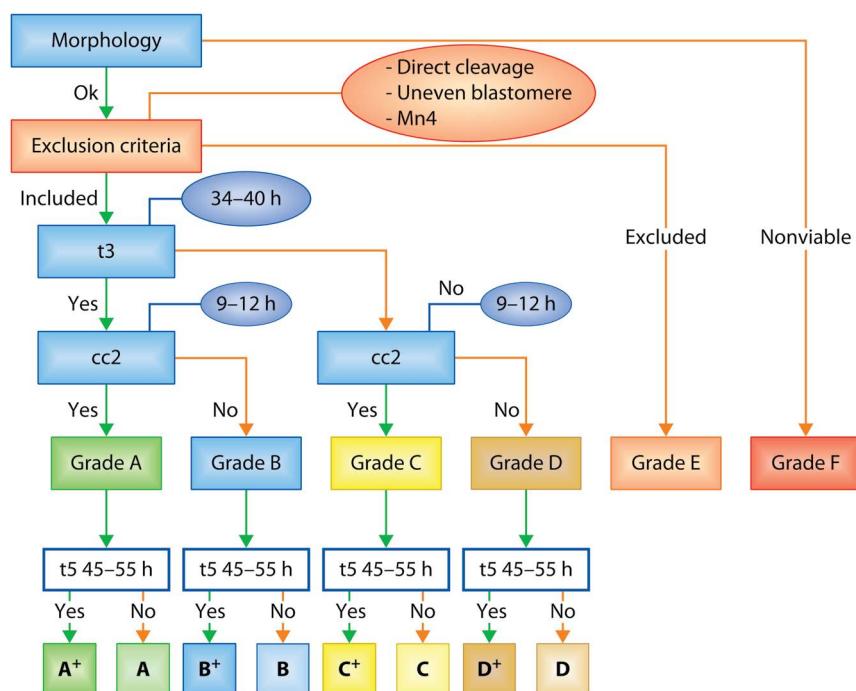


FIGURE 17.8 Revised embryo categorization algorithm. (From [16], reproduced with permission.)

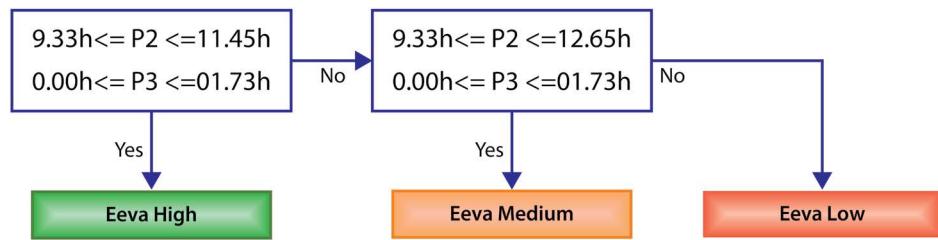


FIGURE 17.9 Modified embryo categorization algorithm known as EEVA II. (Based on data from [56].)

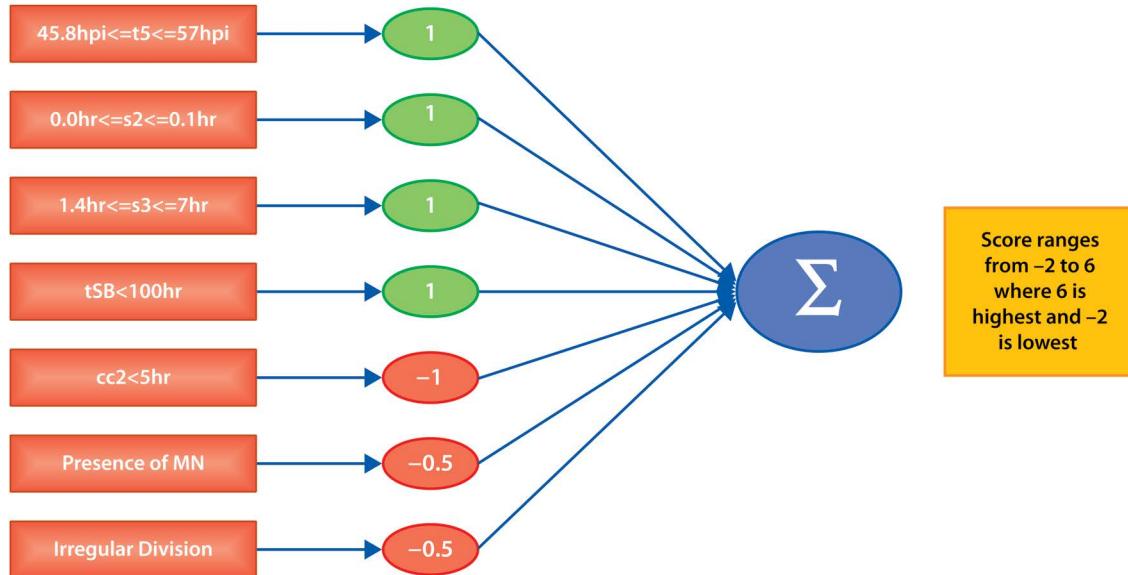


FIGURE 17.10 Goodman algorithm for embryo implantation (described by [62]).

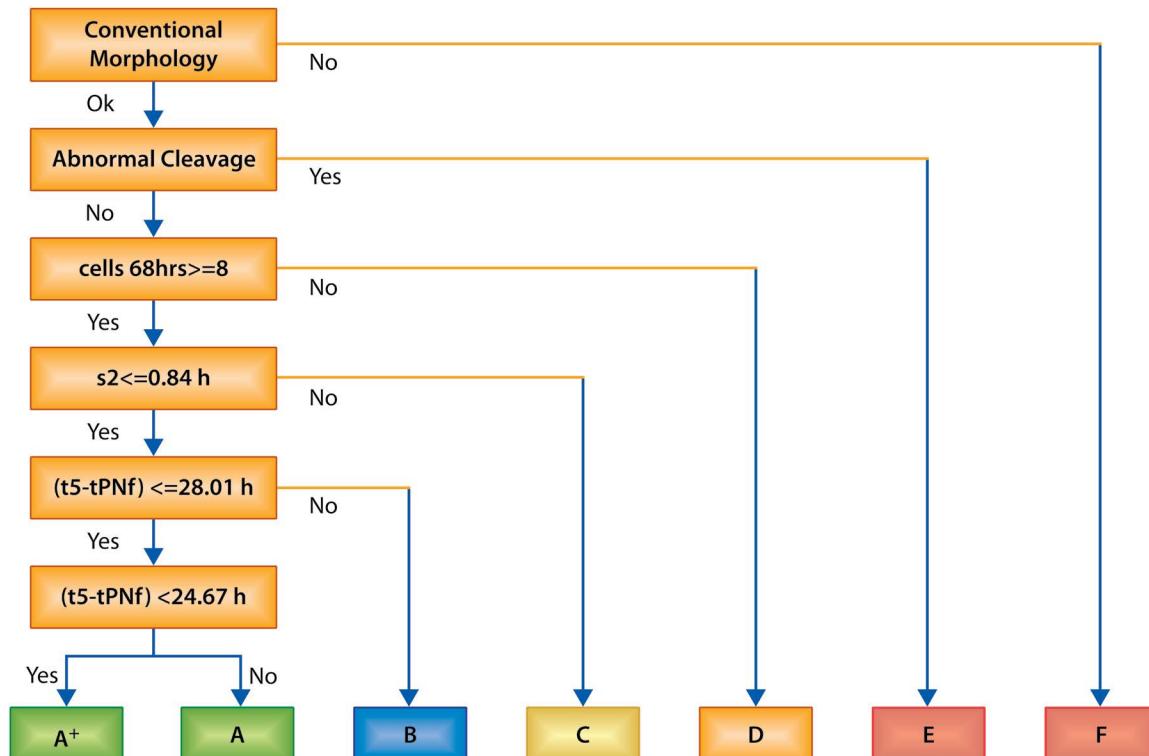


FIGURE 17.11 Liu algorithm for embryo implantation (described by [39]).

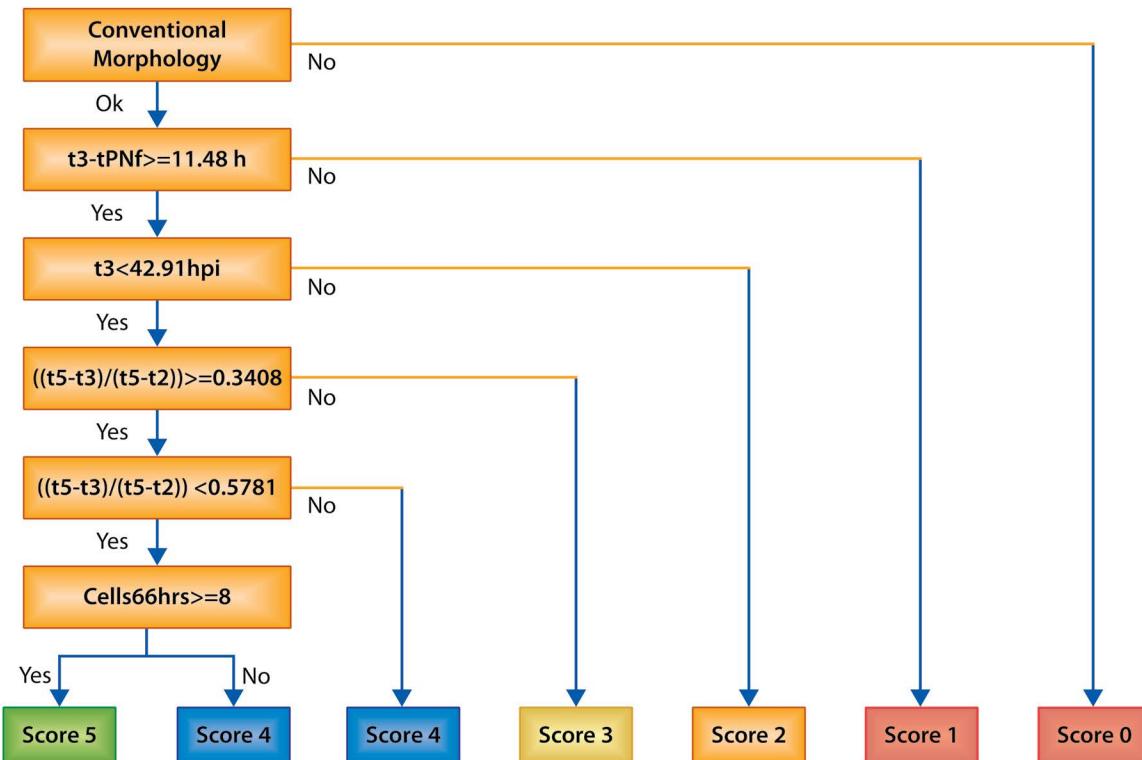


FIGURE 17.12 Petersen algorithm for embryo implantation known as KIDSscore D3 algorithm (described by [63]).

variables— t_5 and s_2 —were considered and not cc_2) performed acceptably. The authors explained that the differences could be the result of variations in oxygen culture conditions, oocyte source (donor cycles vs. autologous cycles), restrictions in the studied population, and/or the stimulation protocols used. The conclusion was that a hierarchical prediction model should not be used universally in an unselected population; it should be centre specific.

The combination of technologies may be the key to improving results in the future. Dominguez et al. [59] combined proteomics and time-lapse analysis of implanted ($n = 16$) and non-implanted ($n = 12$) embryos. After logistic regression analysis, the model identified the presence or absence of protein interleukin-6 and the duration of cc_2 as the most relevant embryo features. Based on these results, the authors developed a hierarchical model (Figure 17.13) based on these two variables, classifying embryos

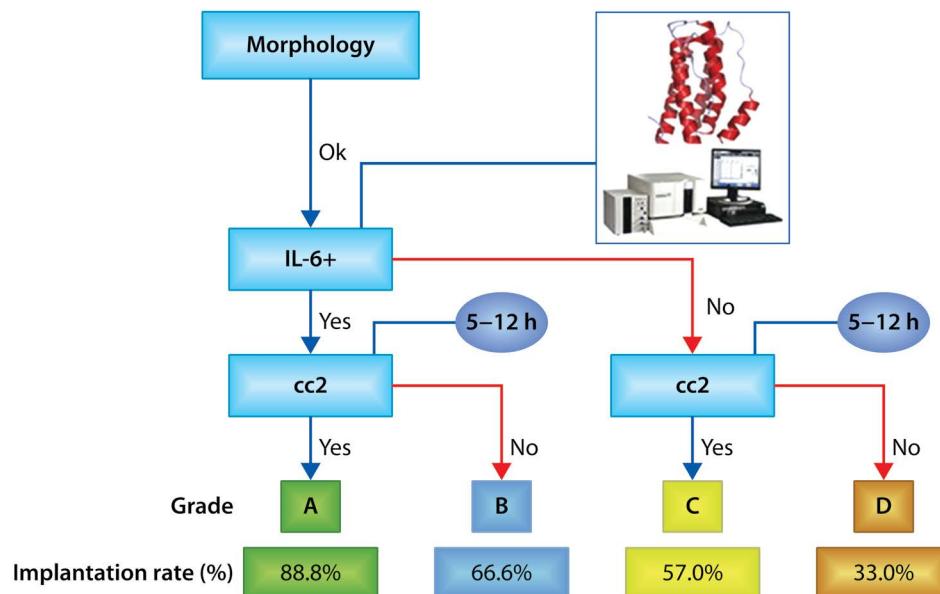


FIGURE 17.13 Combined embryo categorization algorithm. (From [59], reproduced with permission.)

TABLE 17.6 Known Implantation Algorithms that Are Widely Used

Known Model	Score Ranges	Kinetic Parameter	Type of Model
Meseguer Model	A+ – F (10)	t5, s2, cc2, UN 2 cell, MN 4 cell, DC 1–3 cells	Hierarchical
Conaghan Model (EEVA I)	EEVA low, EEVA high	P2, P3	Selection/deselection
VerMileya Model (EEVA II)	EEVA low, EEVA medium, EEVA high	P2, P3	Selection/deselection
EEVA Xtend	1 (highest) – 5 (lowest)	Egg age, cell count, and post P3 analysis	Selection/deselection/hierarchical
Basile Model	A+ – F (10)	cc2, t3, t5, UN 2 cell, MN 4 cell, DC 1–3 cells	Hierarchical
Liu Model	A+ – F	t5, tPNf, s2, cells 68 h, abnormal cleavage	Hierarchical
Motato Model	A–D	T5, t8, tEB	Hierarchical
Goodman Model	–2–4	cc2, MN, IRRD, t5, s2, s3, tSB	Hierarchical
Milewski Model	-		Based on quartile
Petersen	0–5	Not2PN, t2, t3, t5, cells 66hr	Hierarchical
KIDSscore D3 (different version)	0–5	Not2PN, tPNf, t2, t3, t4, t5, t8, cells 66hr	Hierarchical
KIDSscore D5 V2	0–10	Not2PN, t2, t3, t4, t5, tB, tE	Hierarchical (algorithm not shared)
KIDSscore D5 V3 (Different version)	0–10	Not2PN, t2, t3, t4, t5, tB, TCM, TE	Hierarchical (algorithm not shared)
iDA Score	0–10		ANN

into four categories of A–D. Implantation rates are expected to decrease as we move on from A to D as observed in this study (A = 88.8%, B = 66.6%, C = 57%, and D = 33%) [59].

In studies from Kovacic et al. and Ebner et al. observed during warmed blastocyst transfer, kinetic parameter related to blastocyst expansion has been linked to implantation potential [67, 68].

The main algorithms that are widely used are described in Table 17.6.

Aneuploidy studies and where are we

Logically, transferring only euploid embryos should increase live birth rates through increased implantation rates and/or decreased miscarriage rates. However, so far this has been difficult to demonstrate in practice. The correlation between euploidy and embryo kinetics has been studied as well (Table 17.6).

In 2010, Wong et al. [19] collected single embryos for gene expression analysis and revealed that embryos with P1, P2, and P3 outside of the optimal ranges exhibited abnormal RNA patterns for embryo cytokinesis, microRNA biogenesis, and maternal mRNA reserve, suggesting that embryo fate may be predetermined and inherited very early in development (by the 4-cell stage).

Chavez et al. [72] subsequently observed that euploid embryos clustered tightly in the P1, P2, or P3 window, which was predictive of blastocyst formation according to Wong et al.'s study [19]. Performing further molecular analysis, the authors discovered that fragmentation dynamics, together with P1, P2, and P3, could potentially distinguish euploid from aneuploid embryos at the 4-cell stage, considering that the fragments contained nuclear DNA, kinetochore proteins, and whole chromosomes as detected by fluorescence *in situ* hybridization.

In 2013, Campbell et al. [73] elaborated an aneuploidy risk model based on the differences of tSB and tB between euploid and aneuploid embryos that had undergone TE biopsy. The model included three categories: low risk, tB <122.9 hpi and tSB <96.2 hpi; medium risk, tB <122.9 hpi and tSB 96.2 hpi;

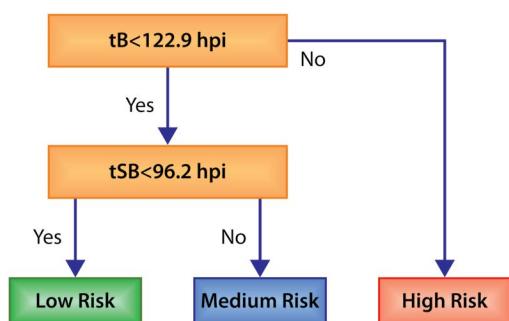
and high risk, tB 122.9 hpi [52]. The same group in a different study [74] applied this model to evaluate its effectiveness and potential clinical impact for unselected IVF patients without undergoing pre-implantation genetic screening after analysing KID embryos. The study revealed significant differences in fetal heart rate (72.7, 25.5, and 0 beats per minute) and live birth rate (61.1%, 19.2%, and 0%) between the three categories low, medium, and high, respectively. This demonstrates that time-lapse imaging using defined morphokinetic data classifies human pre-implantation embryos according to their risk of aneuploidy without performing a biopsy and pre-implantation genetic screening, and that this correlates well with clinical outcomes (Figure 17.14).

In the following year, Basile et al. [75] also correlated morphokinetics with embryo aneuploidy based on 77 patients undergoing genetic screening due to recurrent miscarriage or implantation failure. In this case, embryo biopsy was performed on day 3 of development and the total number of embryos analysed was 504. A logistic regression analysis was used to select and organize which observed timing events (expressed as binary variables inside or outside the optimal range) were most relevant to selecting embryos with higher probabilities of being chromosomally normal. The model identified t5 – t2 (odds ratio [OR] = 2.853, 95% CI = 1.763–4.616) followed by cc3 (OR = 2.095, 95% CI = 1.356–3.238) as the most relevant variables related to normal chromosomal content. An algorithm for embryo selection based on these two variables classified embryos from A to D (Figure 17.15) with significant differences in the percentages of normal embryos as we move on from A to D. More specifically, A = 35.9%, B = 26.4%, C = 12.1%, and D = 9.8% ($p < 0.001$).

As opposed to the previous studies, Rienzi et al. [78] reported no correlation at all between 16 commonly detected morphokinetic parameters and embryo ploidy. This was a longitudinal cohort study conducted using 455 blastocysts from 138 patients at increased risk of aneuploidy because of advance maternal age, history of unsuccessful IVF treatments, or both. The analysed parameters included t2, t3, t4, t5, t8, cc1, cc2, s2, s3, cc3/cc2,

TABLE 17.7 Studies Correlating Euploidy and Embryo Kinetics

Author	Study Design	N	TL System	Biopsy Day	PDG Technology	Parameters with Significant Differences Found
Chavez et al. (2012) [72]	Prospective observational	75	Custom-built microscope	D3	aCGH	P1, P2, P3, and fragmentation
Campbell et al. (2013) [73]	Retrospective cohort	98	Embryoscope	D5	aCGH/SNP array	tSB and tB
Campbell et al. (2013) [74]	Retrospective cohort	88	Embryoscope	D5	aCGH/SNP array	tSB and tB
Basile et al. (2014) [75]	Retrospective cohort	504	Embryoscope	D3	aCGH	t5 – t2 and cc3
Kramer et al. (2014) [76]	Retrospective cohort	149	Embryoscope	D5	aCGH	None
Yang et al. (2014) [77]	Prospective	285	Embryoscope	D5	aCGH	None
Rienzi et al. (2015) [78]	Longitudinal cohort	455	Embryoscope	D5	CCS	None
Chawla et al. (2015) [79]	Retrospective cohort	460	Embryoscope	D3	aCGH	tPNf, t2, t5, cc2, cc3, t5 – t2
Vera-Rodriguez et al. (2015) [80]	Prospective observational	85	EEVA	D3	aCGH	Time between PN disappearance and the start of 1st cytokinesis; 3 to 4 cell
Minasi et al. (2016) [81]	Retrospective cohort	1730/928 cultured in TLT	Embryoscope	D5	aCGH	tSB, tB, tEB, tHB
Balakier et al. (2016) [82]	Retrospective cohort	2441/607 with PGS	Embryoscope	D5	aCGH	NA
Patel et al. (2016) [83]	Retrospective cohort	167	Embryoscope	D3	aCGH	None
Zhan et al. (2016) [40]	Retrospective observational study	1434	Embryoscope	D3/D5/D6	aCGH	DUC
Mumusoglu et al. (2017) [84]	Retrospective cohort	415	Embryoscope	D5	aCGH	t9, tM, tSB, tB, tEB
Del Carmen Nogales et al. (2017) [85]	Retrospective cohort	485	Embryoscope	D3	aCGH	t3, t5 – t2
Zhang et al. (2017) [86]	Retrospective study	256	Embryoscope	D5/6	aCGH	None
Desai et al. (2018) [43]	Retrospective study	767	Embryoscope	D5/6	aCGH	2+ dysmorphism (MN, RC, IDC, DUC), tEB, tSB
Huang et al. (2019) [87]	Retrospective observational study	188	Embryoscope	D5/6	-	Average blastocyst expansion rate
Pennetta et al. (2021) [45]	Retrospective cohort study	287	Embryoscope	D3	-	tPNa



t5–t2, syngamy, tSB, tSC, and tB. Apart from Reinzi, Patel et al., Kramer et al., Yang et al., and Zhang et al. were also not able to correlate aneuploidy detection with kinetic parameters [76, 77, 82–84].

In 2015, two studies observed correlations between embryo kinetics and euploidy. The first one, reported by Chawla et al. [79], identified tPNf, t2, t5, cc2, cc3, and t5–t2 as parameters that significantly differed between chromosomally normal and abnormal embryos. The second one, by Vera-Rodriguez et al. [80], combined chromosomal assessment and single-cell quantitative reverse transcription polymerase chain reaction (RT-qPCR) to simultaneously obtain information from all the blastomeres of human embryos until approximately the 8-cell stage (n = 85). According to their results, the chromosomal status of aneuploid

FIGURE 17.14 Campbell algorithm for ploidy detection (described by [73]).

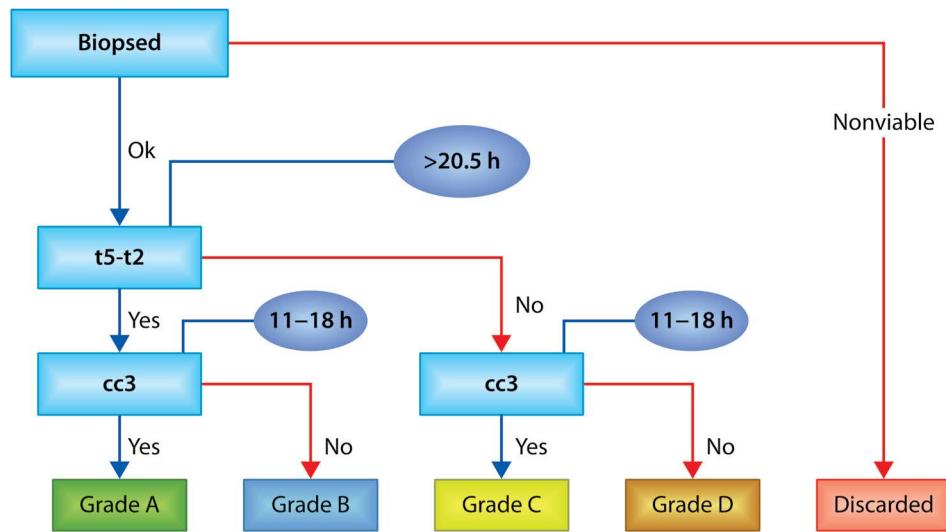


FIGURE 17.15 Embryo selection algorithm. (From [75], reproduced with permission.)

embryos ($n = 26$) correlates with significant differences in the duration of the first mitotic phase when compared with euploid embryos ($n = 28$). Moreover, gene expression profiling in this study suggested that a subset of genes is differentially expressed in aneuploid embryos during the first 30 hours of development.

In 2017, Mumusoglu et al. found statistically significant difference in the kinetic parameter ($t9$, tM , tSB , tB , tEB) of euploid and aneuploid blastocyst through multilevel mixed effect logistic regression analysis [84]. In same year, Del Carmen et al. developed an aneuploidy detection algorithm based on kinetic parameters [85] (Figure 17.16).

In 2018, Desai et al. found more than two dysmorphisms (MN, RC, IDC, DUC) can increase the aneuploidy [43]. In a recent study from Pennetta et al. [86] observed tPNa is linked to the ploidy detection. In the last decade, many studies have tried to correlate different kinetic markers and events to ploidy detection but still PGT-A is standard for ploidy detection. As of now, we stand at a point where TLT can only help to select a better embryo for

implantation if the patient is not at a risk for aneuploidy; but for the patient at risk, TLT technology cannot be trusted from the point of aneuploidy detection, PGT-A needs to be done. In recent years, AI seems to be promising to help the ploidy detection, but a lot of studies, specifically RCT, need to be done.

Time-lapse technology and review

In the last decade, TLT system went through many changes from the point of embryo assessment. The continuous monitoring of embryos provided enough data to embryologist and technology providers to analyse this data. Artificial intelligence and many other statistical approaches have been used to understand the data to improve the IVF process, from selection of embryo to ploidy detection. The development of TLT from manual annotation to fully automatic annotation is a remarkable achievement. Different scoring methods and algorithms have been developed in the last decade using machine learning approaches based on

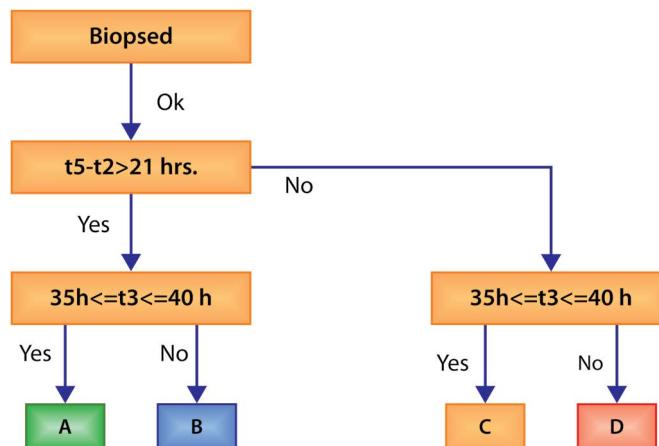


FIGURE 17.16 Del Carmen algorithm for ploidy detection (described by [85]).

TABLE 17.8 Review Studies on Morphokinetics.

Review	Title
Kirkegaard et al. 2012 [71]	Time-Lapse Monitoring as a Tool for Clinical Embryo Assessment
Chen et al. 2013 [89]	Biomarkers Identified with Time-Lapse Imaging: Discovery, Validation, and Practical Application
Kaser et al. 2014 [10]	Clinical Outcomes Following Selection of Human Preimplantation Embryos with Time-Lapse Monitoring: A Systematic Review
Kirkegaard et al. 2014 [11]	Choosing the Best Embryo by Time Lapse versus Standard Morphology
Polanski et al. 2014 [90]	Time-Lapse Embryo Imaging for Improving Reproductive Outcomes: Systematic Review and Meta-Analysis
Gardner et al. 2015 [4]	Diagnosis of Human Preimplantation Embryo Viability
Lundin et al. 2015 [91]	Quality Control and Standardization of Embryo Morphology Scoring and Viability Markers
Racowsky et al. 2015 [92]	A Critical Appraisal of Time-Lapse Imaging for Embryo Selection: Where Are We and Where Do We Need to Go?
Chen et al. 2017 [93]	Does Time-Lapse Imaging Have Favourable Results for Embryo Incubation and Selection Compared with Conventional Methods in Clinical In Vitro Fertilization? A Meta-Analysis and Systematic Review of Randomized Controlled Trials
Milewski et al. 2017 [94]	Time-Lapse Imaging of Cleavage Divisions in Embryo Quality Assessment
Pribenszky et al. 2017 [95]	Time-Lapse Culture with Morphokinetic Embryo Selection Improves Pregnancy and Live Birth Chances and Reduces Early Pregnancy Loss: A Meta-Analysis
Zaninovic et al. 2017 [96]	Assessment of Embryo Morphology and Developmental Dynamics by Time-Lapse Microscopy: Is There a Relation to Implantation and Ploidy?
Adolfsson et al. 2018 [97]	Morphology vs Morphokinetics: A Retrospective Comparison of Interobserver and Intra-Observer Agreement between Embryologists on Blastocysts with Known Implantation Outcome
Reignier et al. 2018 [98]	Can Time-Lapse Parameters Predict Embryo Ploidy? A Systematic Review
Armstrong et al. 2019 [99]	Time-Lapse Systems for Embryo Incubation and Assessment in Assisted Reproduction
Gallego et al. 2019 [100]	Time-Lapse Imaging: The State of the Art
Apter et al. 2020 [17]	Good Practice Recommendations for the Use of Time-Lapse Technology
Fernandez et al. 2020 [101]	Artificial Intelligence in the IVF Laboratory: Overview through the Application of Different Types of Algorithms for the Classification of Reproductive Data
Liu et al. 2020 [102]	Between-Laboratory Reproducibility of Time-Lapse Embryo Selection Using Qualitative and Quantitative Parameters: A Systematic Review and Meta-Analysis
Lundin et al. 2020 [103]	Time-Lapse Technology for Embryo Culture and Selection
Minasi et al. 2020 [104]	The Clinical Use of Time-Lapse in Human-Assisted Reproduction
Sciorio et al. 2021 [105]	Focus on Time-Lapse Analysis: Blastocyst Collapse and Morphometric Assessment as New Features of Embryo Viability
Kragh et al. 2021 [106]	Embryo Selection with Artificial Intelligence: How to Evaluate and Compare Methods?
Dimitriadi et al. 2022 [107]	Artificial Intelligence in the Embryology Laboratory: A Review

blastocyst formation, implantation (KID score), and fetal heart-beat (iDA score).

A lot of the reviews, opinions, and future directions regarding time-lapse that have been published in last decade are mentioned in Table 17.8.

Conclusion

Static observations obtained from standard microscopes have contributed significantly to our knowledge of embryo development; however, it is becoming more challenging to identify embryos with the highest implantation potential due to the static and notoriously subjective character of this type of morphological evaluation. The study of embryo kinetics through time-lapse technology has given rise to new markers for embryo selection, representing a new and excitingly powerful tool for viewing cellular activity and embryogenesis in a coherent and uninterrupted manner that is otherwise not available through standard microscopy. The current chapter presents an overview of the most recent studies that describe the use of this new technology in the IVF laboratory. Currently, TLT has been introduced into the IVF

laboratory as a routine procedure, but many laboratories are still using standard incubators as a routine procedure because of cost and other factors. TLT provides the safest and most stable environment for the embryo culture and continuous embryo monitoring, which allowed us to identify many more undetected and unknown parameters of embryonic development. TLT provides a great hope for the future for non-invasive markers in the detection of aneuploidy, embryo abnormality, embryo selectivity, etc., with a combination of AI, metabolomics, proteomics, and secretomics.

References

1. Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remoh J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod*. 2011;26(10):2658–71.
2. Mio Y, Maeda K. Time-lapse cinematography of dynamic changes occurring during *in vitro* development of human embryos. *Am J Obstet Gynecol*. 2008;199(6):660.e1–660.e5.
3. Balaban B, Brison D, Calderón G, Catt J, Conaghan J, Cowan L, et al. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Hum Reprod*. 2011;26(6):1270–83.

4. Gardner DK, Meseguer M, Rubio C, Treff NR. Diagnosis of human preimplantation embryo viability. *Hum Reprod Update*. 2015;21(6):727–47.
5. Baxter Bendus AE, Mayer JF, Shipley SK, Catherino WH. Interobserver and intraobserver variation in day 3 embryo grading. *Fertil Steril*. 2006;86(6):1608–15.
6. Paternot G, Wetsels AM, Thonon F, Vansteenberghe A, Willemen D, Devroe J, et al. Intra- and interobserver analysis in the morphological assessment of early stage embryos during an IVF procedure: A multicentre study. *Reprod Biol Endocrinol*. 2011;9(1):1–5.
7. Sundvall L, Ingerslev HJ, Breth Knudsen U, Kirkegaard K. Inter- and intra-observer variability of time-lapse annotations. *Hum Reprod*. 2013;28(12):3215–21.
8. Arce JC, Ziebe S, Lundin K, Janssens R, Helmgård L, Sørensen P. Interobserver agreement and intraobserver reproducibility of embryo quality assessments. *Hum Reprod*. 2006;21(8):2141–8.
9. Scott L. The biological basis of non-invasive strategies for selection of human oocytes and embryos. *Hum Reprod Update*. 2003;9(3):237–49.
10. Kaser DJ, Racowsky C. Clinical outcomes following selection of human preimplantation embryos with time-lapse monitoring: A systematic review. *Hum Reprod Update*. 2014;20(5):617–31.
11. Kirkegaard K, Ahlström A, Ingerslev HJ, Hardarson T. Choosing the best embryo by time lapse versus standard morphology. *Fertil Steril*. 2015;103(2):323–32.
12. Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, Requena A. Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: A retrospective cohort study. *Fertil Steril*. 2012;98(6):1481–1489.e10.
13. Zhang JQ, Li XL, Peng Y, Guo X, Heng BC, Tong GQ. Reduction in exposure of human embryos outside the incubator enhances embryo quality and blastulation rate. *Reprod Biomed Online*. 2010;20(4):510–5.
14. Payne D, Flaherty SP, Barry MF, Matthews CD. Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. *Hum Reprod*. 1997;12(3):532–41.
15. Basile N, Caiazza M, Meseguer M. What does morphokinetics add to embryo selection and in-vitro fertilization outcomes? *Curr Opin Obstet Gynecol*. 2015;27(3):193–200.
16. Herrero J, Meseguer M. Selection of high potential embryos using time-lapse imaging: The era of morphokinetics. *Fertil Steril*. 2013;99(4):1030–4.
17. Apter S, Ebner T, Freour T, Guns Y, Kovacic B, le Clef N, et al. Good practice recommendations for the use of time-lapse technology. *Hum Reprod Open*. 2020;2020(2):1–26.
18. Ciray HN, Campbell A, Agerholm IE, Aguilar J, Chamayou S, Esbert M, et al. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. *Hum Reprod*. 2014;29(12):2650–60.
19. Wong CC, Loewke KE, Bossert NL, Behr B, de Jonge CJ, Baer TM, et al. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol*. 2010;28(10):1115–21.
20. Hashimoto S, Kato N, Saeki K, Morimoto Y. Selection of high-potential embryos by culture in poly(dimethylsiloxane) microwells and time-lapse imaging. *Fertil Steril*. 2012;97(2):332–7.
21. Coticchio G, Renzini MM, Novara PV, Lain M, de Ponti E, Turchi D, et al. Focused time-lapse analysis reveals novel aspects of human fertilization and suggests new parameters of embryo viability. *Hum Reprod*. 2018;33(1):23–31.
22. Cruz M, Garrido N, Herrero J, Pérez-Cano I, Muñoz M, Meseguer M. Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reprod Biomed Online*. 2012;25(4):371–81.
23. Dal Canto M, Coticchio G, Mignini Renzini M, de Ponti E, Novara PV, Brambillasca F, et al. Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation. *Reprod Biomed Online*. 2012;25(5):474–80.
24. Chamayou S, Patrizio P, Storaci G, Tomaselli V, Alecci C, Ragolia C, et al. The use of morphokinetic parameters to select all embryos with full capacity to implant. *J Assist Reprod Genet*. 2013;30(5):703–10.
25. Kahraman S, Çetinkaya M, Pirkevi C, Yelke H, Kumtepe Y. Comparison of blastocyst development and cycle outcome in patients with eSET using either conventional or time lapse incubators. A Prospective Study of Good Prognosis Patients. *Journal of Reproductive Biotechnology and Fertility*. 2012;3(2):55–61. <http://dx.doi.org/10.1177/205891581200300204>
26. Lemmen JG, Agerholm I, Ziebe S. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. *Reprod Biomed Online*. 2008;17(3):385–91.
27. Hlinka D, Kalatová B, Uhrinová I, Dolinská S, Rutarová J, Řezáčová J, et al. Time-lapse cleavage rating predicts human embryo viability. *Physiol Res*. 2012;61(5):513–25.
28. Kirkegaard K, Kesmodel US, Hindkjaer JJ, Ingerslev HJ. Time-lapse parameters as predictors of blastocyst development and pregnancy outcome in embryos from good prognosis patients: A prospective cohort study. *Hum Reprod*. 2013;28(10):2643–51.
29. Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boosanfar R, et al. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: Results from a prospective multicenter trial. *Fertil Steril*. 2013;100(2).
30. Athayde Wirka K, Chen AA, Conaghan J, Ivani K, Gvakharia M, Behr B, et al. Atypical embryo phenotypes identified by time-lapse microscopy: High prevalence and association with embryo development. *Fertil Steril*. 2014;101(6):1637–48.e1–5.
31. Kirkegaard K, Campbell A, Agerholm I, Bentin-Ley U, Gabrielsen A, Kirk J, et al. Limitations of a time-lapse blastocyst prediction model: A large multicentre outcome analysis. *Reprod Biomed Online*. 2014;29(2):156–8.
32. Desai N, Ploskonka S, Goodman LR, Austin C, Goldberg J, Falcone T. Analysis of embryo morphokinetics, multinucleation and cleavage anomalies using continuous time-lapse monitoring in blastocyst transfer cycles. *Reprod Biol Endocrinol*. 2014;12(1):54.
33. Çetinkaya M, Pirkevi C, Yelke H, Colakoglu YK, Atayurt Z, Kahraman S. Relative kinetic expressions defining cleavage synchronicity are better predictors of blastocyst formation and quality than absolute time points. *J Assist Reprod Genet*. 2015;32(1):27–35.
34. Yang ST, Shi JX, Gong F, Zhang SP, Lu CF, Tan K, et al. Cleavage pattern predicts developmental potential of day 3 human embryos produced by IVF. *Reprod Biomed Online*. 2015;30(6):625–34.
35. Milewski R, Kuć P, Kuczyńska A, Stankiewicz B, Łukaszuk K, Kuczyński W. A predictive model for blastocyst formation based on morphokinetic parameters in time-lapse monitoring of embryo development. *J Assist Reprod Genet*. 2015;32(4):571.
36. Storr A, Venetis CA, Cooke S, Susetio D, Kilani S, Ledger W. Morphokinetic parameters using time-lapse technology and day 5 embryo quality: A prospective cohort study. *J Assist Reprod Genet*. 2015;32(7):1151.
37. Motato Y, de los Santos MJ, Escriba MJ, Ruiz BA, Remohí J, Meseguer M. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertil Steril*. 2016;105(2):376–384.e9.
38. Mizobe Y, Oya N, Iwakiri R, Yoshida N, Sato Y, Miyoshi K, et al. Effects of early cleavage patterns of human embryos on subsequent in vitro development and implantation. *Fertil Steril*. 2016;106(2):348–353.e2.
39. Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse deselection model for human day 3 in vitro fertilization embryos: The combination of qualitative and quantitative measures of embryo growth. *Fertil Steril*. 2016;105(3):656–662.e1.

40. Zhan Q, Ye Z, Clarke R, Rosenwaks Z, Zaninovic N. Direct unequal cleavages: Embryo developmental competence, genetic constitution and clinical outcome. *PLoS One.* 2016;11(12):e0166398.
41. Mizobe Y, Tokunaga M, Oya N, Iwakiri R, Yoshida N, Sato Y, et al. Synchrony of the first division as an index of the blastocyst formation rate during embryonic development. *Reprod Med Biol.* 2018;17(1):64–70.
42. Fishel S, Campbell A, Montgomery S, Smith R, Nice L, Duffy S, et al. Time-lapse imaging algorithms rank human preimplantation embryos according to the probability of live birth. *Reprod Biomed Online.* 2018;37(3):304–13.
43. Desai N, Goldberg JM, Austin C, Falcone T. Are cleavage anomalies, multinucleation, or specific cell cycle kinetics observed with time-lapse imaging predictive of embryo developmental capacity or ploidy? *Fertil Steril.* 2018;109(4):665–74.
44. Lagalla C, Coticchio G, Sciajno R, Tarozzi N, Zacà C, Borini A. Alternative patterns of partial embryo compaction: Prevalence, morphokinetic history and possible implications. *Reprod Biomed Online.* 2020;40(3):347–54.
45. Pennetta F, Lagalla C, Sciajno R, Tarozzi N, Nadalini M, Zacà C, et al. The association of kinetic variables with blastocyst development and ploidy status. *J Reprod Infertil.* 2021;22(3):159.
46. María C, Nicolás G, Inmaculada P-C, Niels R, Manuel M, Marcos M. Comparative study of embryo quality, blastocyst and ongoing pregnancy rates in oocyte donation patients sharing embryoscope and standard incubator. *Fertil Steril.* 2010;94(4):S78.
47. Cruz M, Gadea B, Garrido N, Pedersen KS, Martínez M, Pérez-Cano I, et al. Embryo quality, blastocyst and ongoing pregnancy rates in oocyte donation patients whose embryos were monitored by time-lapse imaging. *J Assist Reprod Genet.* 2011;28(7):569.
48. Rubio I, Galán A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, et al. Clinical validation of embryo culture and selection by morphokinetic analysis: A randomized, controlled trial of the EmbryoScope. *Fertil Steril.* 2014;102(5):1287–1294.e5.
49. Siristatidis C, Komitopoulou MA, Makris A, Sialakouma A, Botzaki M, Mastorakos G, et al. Morphokinetic parameters of early embryo development via time lapse monitoring and their effect on embryo selection and ICSI outcomes: A prospective cohort study. *J Assist Reprod Genet.* 2015;32(4):563–70.
50. Azzarello A, Hoest T, Mikkelsen AL. The impact of pronuclei morphology and dynamicity on live birth outcome after time-lapse culture. *Hum Reprod.* 2012;27(9):2649–57.
51. Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escrivá MJ, et al. Limited implantation success of direct-cleaved human zygotes: A time-lapse study. *Fertil Steril.* 2012;98(6):1458–63.
52. Fréour T, Dessolle L, Lammers J, Lattes S, Barrière P. Comparison of embryo morphokinetics after in vitro fertilization-intracytoplasmic sperm injection in smoking and nonsmoking women. *Fertil Steril.* 2013;99(7):1944–50.
53. Aguilar J, Motato Y, Escrivá MJ, Ojeda M, Muñoz E, Meseguer M. The human first cell cycle: Impact on implantation. *Reprod Biomed Online.* 2014;28(4):475–84.
54. Basile N, Vime P, Florensa M, Aparicio Ruiz B, García Velasco JA, Remohí J, et al. The use of morphokinetics as a predictor of implantation: A multicentric study to define and validate an algorithm for embryo selection. *Hum Reprod.* 2015;30(2):276–83.
55. Ergin EG, Çalışkan E, Yalçınkaya E, Öztel Z, Çökelez K, Özay A, et al. Frequency of embryo multinucleation detected by time-lapse system and its impact on pregnancy outcome. *Fertil Steril.* 2014;102(4):1029–1033.e1.
56. Vermilyea MD, Tan L, Anthony JT, Conaghan J, Ivani K, Gvakharia M, et al. Computer-automated time-lapse analysis results correlate with embryo implantation and clinical pregnancy: A blinded, multi-centre study. *Reprod Biomed Online.* 2014;29(6):729–36.
57. Fréour T, le Fleuter N, Lammers J, Spingart C, Reignier A, Barrière P. External validation of a time-lapse prediction model. *Fertil Steril.* 2015;103(4):917–22.
58. Marcos J, Pérez-Albalá S, Mifsud A, Molla M, Landeras J, Meseguer M. Collapse of blastocysts is strongly related to lower implantation success: A time-lapse study. *Hum Reprod.* 2015;30(11):2501–8.
59. Dominguez F, Meseguer M, Aparicio-Ruiz B, Piqueras P, Quiñonero A, Simón C. New strategy for diagnosing embryo implantation potential by combining proteomics and time-lapse technologies. *Fertil Steril.* 2015;104(4):908–14.
60. Adamson GD, Abusief ME, Palao L, Witmer J, Palao LM, Gvakharia M. Improved implantation rates of day 3 embryo transfers with the use of an automated time-lapse-enabled test to aid in embryo selection. *Fertil Steril.* 2016;105(2):369–375.e6.
61. Wu L, Han W, Zhang X, Wang J, Liu W, Xiong S, et al. A retrospective analysis of morphokinetic parameters according to the implantation outcome of IVF treatment. *Eur J Obstet Gynecol Reprod Biol.* 2016;197:186–90.
62. Goodman LR, Goldberg J, Falcone T, Austin C, Desai N. Does the addition of time-lapse morphokinetics in the selection of embryos for transfer improve pregnancy rates? A randomized controlled trial. *Fertil Steril.* 2016;105(2):275–285.e10.
63. Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. *Hum Reprod.* 2016;31(10):2231–44.
64. Desai N, Ploskonka S, Goodman L, Attaran M, Goldberg JM, Austin C, et al. Delayed blastulation, multinucleation, and expansion grade are independently associated with live-birth rates in frozen blastocyst transfer cycles. *Fertil Steril.* 2016;106(6):1370–8.
65. Carrasco B, Arroyo G, Gil Y, Gómez MJ, Rodríguez I, Barri PN, et al. Selecting embryos with the highest implantation potential using data mining and decision tree based on classical embryo morphology and morphokinetics. *J Assist Reprod Genet.* 2017;34(8):983–90.
66. Mizobe Y, Ezono Y, Tokunaga M, Oya N, Iwakiri R, Yoshida N, et al. Selection of human blastocysts with a high implantation potential based on timely compaction. *J Assist Reprod Genet.* 2017;34(8):991.
67. Ebner T, Oppelt P, Radler E, Allerstorfer C, Habsberger A, Mayer RB, et al. Morphokinetics of vitrified and warmed blastocysts predicts implantation potential. *J Assist Reprod Genet.* 2017;34(2):239–44.
68. Kovačić B, Taborin M, Vlaisavljević V. Artificial blastocoel collapse of human blastocysts before vitrification and its effect on re-expansion after warming – A prospective observational study using time-lapse microscopy. *Reprod Biomed Online.* 2018;36(2):121–9.
69. Viñals Gonzalez X, Odia R, Cawood S, Gaunt M, Saab W, Seshadri S, et al. Contraction behaviour reduces embryo competence in high-quality euploid blastocysts. *J Assist Reprod Genet.* 2018;35(8):1509–17.
70. Bartolacci A, Dal Canto M, Guglielmo MC, Mura L, Brigante C, Mignini Renzini M, et al. Early embryo morphokinetics is a better predictor of post-ICSI live birth than embryo morphology: Speed is more important than beauty at the cleavage stage. *Zygote.* 2021;29(6):495–502.
71. Kirkegaard K, Agerholm IE, Ingerslev HJ. Time-lapse monitoring as a tool for clinical embryo assessment. *Hum Reprod.* 2012;27(5):1277–85.
72. Chavez SL, Loewke KE, Han J, Moussavi F, Colls P, Munne S, et al. Dynamic blastomere behaviour reflects human embryo ploidy by the four-cell stage. *Nat Commun.* 2012;3:1–12. <http://dx.doi.org/10.1038/ncomms2249>
73. Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Hickman CFL. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. *Reprod Biomed Online.* 2013;26(5):477–85.

74. Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Thornton S. Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. *Reprod Biomed Online.* 2013;27(2):140–6.
75. Basile N, Nogales MDC, Bronet F, Florena M, Riqueiros M, Rodrigo L, et al. Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. *Fertil Steril.* 2014;101(3):699–704.
76. Kramer YG, Kofinas JD, Melzer K, Noyes N, McCaffrey C, Buldo-Licciardi J, et al. Assessing morphokinetic parameters via time lapse microscopy (TLM) to predict euploidy: Are aneuploidy risk classification models universal? *J Assist Reprod Genet.* 2014;31(9):1231–42.
77. Yang Z, Zhang J, Salem SA, Liu X, Kuang Y, Salem RD, et al. Selection of competent blastocysts for transfer by combining time-lapse monitoring and array CGH testing for patients undergoing preimplantation genetic screening: A prospective study with sibling oocytes. *BMC Med Genomics.* 2014;7(1):38.
78. Rienzi L, Capalbo A, Stoppa M, Romano S, Maggiulli R, Albricci L, et al. No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: A longitudinal cohort study. *Reprod Biomed Online.* 2015;30(1):57–66.
79. Chawla M, Fakih M, Shunnar A, Bayram A, Hellani A, Perumal V, et al. Morphokinetic analysis of cleavage stage embryos and its relationship to aneuploidy in a retrospective time-lapse imaging study. *J Assist Reprod Genet.* 2015;32(1):69–75.
80. Vera-Rodriguez M, Chavez SL, Rubio C, Reijo Pera RA, Simon C. Prediction model for aneuploidy in early human embryo development revealed by single-cell analysis. *Nat Commun.* 2015;6:7601.
81. Minasi MG, Colasante A, Riccio T, Ruberti A, Casciani V, Scarselli F, et al. Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: A consecutive case series study. *Hum Reprod.* 2016;31(10):2245–54.
82. Balakier H, Sojecki A, Motamedi G, Librach C. Impact of multinucleated blastomeres on embryo developmental competence, morphokinetics, and aneuploidy. *Fertil Steril.* 2016;106(3):608–614.e2.
83. Patel DV, Shah PB, Kotdawala AP, Herrero J, Rubio I, Bunker MR. Morphokinetic behavior of euploid and aneuploid embryos analyzed by time-lapse in embryoscope. *J Hum Reprod Sci.* 2016;9(2):112–8.
84. Mumusoglu S, Yarali I, Bozdag G, Ozdemir P, Polat M, Sokmensuer LK, et al. Time-lapse morphokinetic assessment has low to moderate ability to predict euploidy when patient- and ovarian stimulation-related factors are taken into account with the use of clustered data analysis. *Fertil Steril.* 2017;107(2):413–421.e4.
85. del Carmen Nogales M, Bronet F, Basile N, Martínez EM, Liñán A, Rodrigo L, et al. Type of chromosome abnormality affects embryo morphology dynamics. *Fertil Steril.* 2017;107(1):229–235.e2.
86. Zhang J, Tao W, Liu H, Yu G, Li M, Ma S, et al. Morphokinetic parameters from a time-lapse monitoring system cannot accurately predict the ploidy of embryos. *J Assist Reprod Genet.* 2017;34(9):1173–8.
87. Huang TT, Huang DH, Ahn HJ, Arnett C, Huang CT. Early blastocyst expansion in euploid and aneuploid human embryos: Evidence for a non-invasive and quantitative marker for embryo selection. *Reprod Biomed Online.* 2019;39(1):27–39.
88. Pennetta F, L C, Borini A. Embryo morphokinetic characteristics and euploidy. *Curr Opin Obstet Gynecol.* 2018;30:185–96.
89. Chen AA, Tan L, Suraj V, Reijo Pera R, Shen S. Biomarkers identified with time-lapse imaging: Discovery, validation, and practical application. *Fertil Steril.* 2013;99(4):1035–43.
90. Polanski LT, Coelho Neto MA, Nastri CO, Navarro PA, Ferriani RA, Raine-Fenning N, et al. Time-lapse embryo imaging for improving reproductive outcomes: Systematic review and meta-analysis. *Ultrasound Obstet Gynecol.* 2014;44(4):394–401.
91. Lundin K, Ahlström A. Quality control and standardization of embryo morphology scoring and viability markers. *Reprod Biomed Online.* 2015;31(4):459–71. <http://dx.doi.org/10.1016/j.rbmo.2015.06.026>
92. Racowsky C, Kovacs P, Martins WP. A critical appraisal of time-lapse imaging for embryo selection: Where are we and where do we need to go? *J Assist Reprod Genet.* 2015;32(7):1025–30.
93. Chen M, Wei S, Hu J, Yuan J, Liu F. Does time-lapse imaging have favorable results for embryo incubation and selection compared with conventional methods in clinical in vitro fertilization? A meta-analysis and systematic review of randomized controlled trials. *PLoS ONE.* 2017;12(6):e0178720.
94. Milewski R, Ajduk A. Time-lapse imaging of cleavage divisions in embryo quality assessment. *Reproduction.* 2017;154(2):R37–53.
95. Pribenszky C, Nilselid AM, Montag M. Time-lapse culture with morphokinetic embryo selection improves pregnancy and live birth chances and reduces early pregnancy loss: A meta-analysis. *Reprod Biomed Online.* 2017;35(5):511–20.
96. Zaninovic N, Irani M, Meseguer M. Assessment of embryo morphology and developmental dynamics by time-lapse microscopy: Is there a relation to implantation and ploidy? *Fertil Steril.* 2017;108(5):722–9.
97. Adolfsson E, Andershed AN. Morphology vs morphokinetics: A retrospective comparison of interobserver and intra-observer agreement between embryologists on blastocysts with known implantation outcome. *Jornal Brasileiro de Reproducao Assistida.* 2018;22(3):228–37.
98. Reignier A, Lammers J, Barriere P, Freour T. Can time-lapse parameters predict embryo ploidy? A systematic review. *Reprod Biomed Online.* 2018;36(4):380–7.
99. Armstrong S, Bhide P, Jordan V, Pacey A, Marjoribanks J, Farquhar C. Time-lapse systems for embryo incubation and assessment in assisted reproduction. *Cochrane Data Syst Rev.* 2019;5(5):CD011320.
100. Gallego R, del Remohí J, Meseguer M, Gardner DK. Time-lapse imaging: The state of the art. *Biol Reprod.* 2019;101(6):1146–54.
101. Fernandez EI, Ferreira AS, Cecílio MHM, Chéles DS, de Souza RCM, Nogueira MFG, et al. Artificial intelligence in the IVF laboratory: Overview through the application of different types of algorithms for the classification of reproductive data. *J Assist Reprod Genet.* 2020;37(10):2359–76.
102. Liu Y, Qi F, Matson P, Morbeck DE, Mol BW, Zhao S, et al. Between-laboratory reproducibility of time-lapse embryo selection using qualitative and quantitative parameters: A systematic review and meta-analysis. *J Assist Reprod Genet.* 2020;37(6):1295–302.
103. Lundin K, Park H. Time-lapse technology for embryo culture and selection. *Ups J Med Sci.* 2020;125(2):77–84.
104. Minasi MG, Greco P, Varricchio MT, Barillari P, Greco E. The clinical use of time-lapse in human-assisted reproduction. *Ther Adv Reprod Health.* 2020;14:263349412097692.
105. Sciorio R, Meseguer M. Focus on time-lapse analysis: Blastocyst collapse and morphometric assessment as new features of embryo viability. *Reprod Biomed Online.* 2021;43(5):821–32. <https://doi.org/10.1016/j.rbmo.2021.08.008>
106. Kragh MF, Karstoft H. Embryo selection with artificial intelligence: How to evaluate and compare methods? *J Assist Reprod Genet.* 2021;38(7):1675–89.
107. Dimitriadis I, Zaninovic N, Badiola AC, Bormann CL. Artificial intelligence in the embryology laboratory: A review. *Reprod Biomed Online.* 2022;44(3):435–48. <https://doi.org/10.1016/j.rbmo.2021.11.003>

18

ARTIFICIAL INTELLIGENCE (AI)

Daniella Gilboa

Artificial intelligence (AI) is one of the greatest promises of the near future. By most accounts, it is going to be pertinent to the field of medicine, very significantly and very soon. As a recent editorial in the *Lancet* stated “[a] scenario in which medical information, gathered at the point of care, is analyzed using sophisticated machine algorithms to provide real-time actionable analytics seems to be within touching distance” [1].

A myriad of emerging technologies seems to be flooding hospitals, primary care practices, and the like, but to a large degree human-to-human interactions still drive the healthcare industry. AI is poised to change that—complementing human decision-making by working alongside physicians. At long last, the expert-only approach to medicine is beginning to change, thanks to the massive growth of medical data and the power of AI.

The healthcare industry is digitizing at a rapid rate: health records, medical images, and even discussions about treatment options are all being recorded digitally. In 1950, it took 50 years for the total volume of medical knowledge to double—by 2020, that doubling time is just about 73 days. Deep learning is considered a form of AI, as it bears similarities to human-based learning. The growing wide availability of digitalized data allows deep learning, which can recognize patterns in complex data sets, to revolutionize the way we practice medicine.

The main aspects of human intelligence are quite similar to AI. In the same way that humans gather information, process it, and determine an output, machines can do this as well. Because machines do not have physical senses like people do, the way they gather input differs. AI gathers information through things like speech recognition, visual recognition, and other data sources.

The processing piece of the formula also mimics how human intelligence works. Like the way people acquire memories and build knowledge, machines can create representations of knowledge and databases where information is stored. And, in the same manner that people draw inferences and make decisions, machines can predict, optimize, and determine what the best “next steps” should be in order to accomplish a particular goal.

Just as humans learn, machines can also be “taught.” For instance, supervised machine learning means learning by example: the computer is provided with a data set containing labels that act as answers. Over time the machine can essentially “learn” to differentiate between those labels to produce the correct outcome.

Unsupervised machine learning is like learning by observation. The computer recognizes and identifies certain patterns and subsequently learns how to distinguish groups and patterns on its own [2]. To understand the concept of deep neural networks (DNNs), we should define the basic unit—a neuron. A neuron is a mathematical function which represents a learning unit.

A neural network is a network of functions, meaning all functions (or learning units) and all their inputs and outputs are intertwined and feed each other in order to learn the problem out of a

set of examples (Figure 18.1). A neural network can learn relationships between the features that other algorithms cannot easily discover [3].

While the roots of AI date back more than 80 years from concepts laid out by Alan Turing [5, 6], Warren McCulloch, and Walter Pitts [7], it was not until 2012 that the subtype of deep learning was widely accepted as a viable form of AI. A deep learning neural network consists of digitized inputs, such as an image or speech, which proceed through multiple layers of connected “neurons” that progressively detect features, and ultimately provide an output. The basic DNN architecture is like a club sandwich turned on its side, with an input layer, several hidden layers ranging from 5 to 1000, each responding to different features of the image (like shape or edges), and an output layer. The layers are “neurons,” comprising a neural network. A key differentiating feature of deep learning compared with other subtypes of AI is its autodidactic quality; the neural network is not designed by humans, but rather the number of layers is determined by the data itself. There are many types of DNNs and learning, including convolutional, recurrent, generative adversarial, reinforcement, representation, and transfer [8, 9].

Deep learning algorithms have been the backbone of computer performance. They exceed human ability in multiple games and are largely responsible for the exceptional progress in autonomous cars (Figure 18.2). Notably, except in the cases of games and self-driving cars, a major limitation to interpretation of claims reporting superhuman performance of these algorithms is that analytics are performed on previously generated data rather than prospectively in real-world clinical conditions. Furthermore, the lack of large data sets of carefully annotated images has been limiting across various disciplines in medicine.

Recent advances in generative AI (GenAI) have opened a new perspective for AI.

GenAI is a type of AI model that can create a wide variety of content such as text, images, videos, audios, and 3D models. It does so by using large language models (LLMs) to train on very large amounts of data, and then uses this knowledge to generate new and unique outputs. GenAI primarily differs from previous forms of AI or analytics because it can generate new content, often in “unstructured” forms.

LLMs are recent advances in deep learning models that work on human languages. An LLM is a trained deep-learning model that understands and generates text in a human-like fashion. Behind the scenes, it is a large transformer model that does all the magic. A transformer model is a neural network that learns context and meaning by tracking relationships in sequential data, like the words in a sentence.

Transformer models apply an evolving set of mathematical techniques called the attention mechanism, which allows us to see the entire sentence (or even the paragraph) at once rather than one word at a time. This allows the transformer model to understand the context of a word better [10].

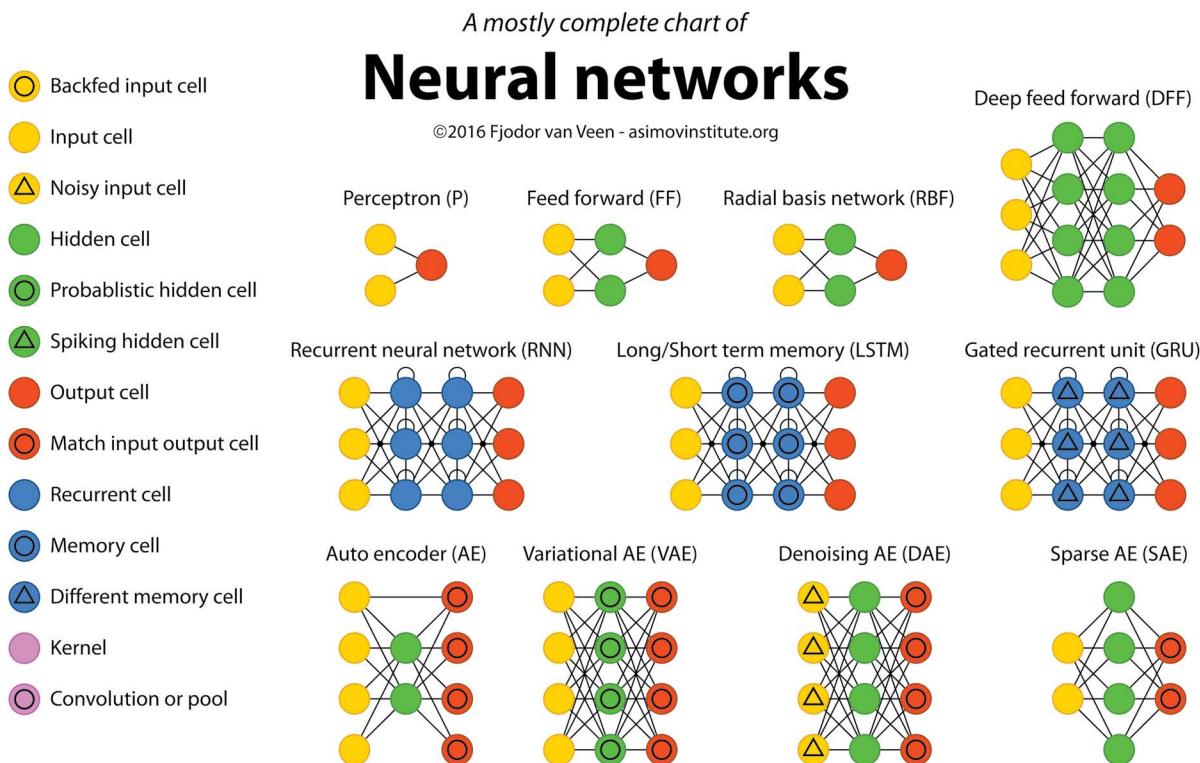


FIGURE 18.1 Examples of neural networks. (From [4], with permission.)

AI

- Device mimics cognitive functions
- Since 1950s

Machine learning

- Algorithms that improve as they are exposed to more data
- Since 1980s

Deep learning

- Artificial neural networks structured in multiple layers to decode imaging raw data
- Since 2010s

The speed at which GenAI technology is developing is amazing. ChatGPT was released in November 2022. Four months later, OpenAI released a new LLM called GPT-4 with markedly improved capabilities. In May 2023, Google announced several new features powered by GenAI, including Search Generative Experience and a new LLM called PaLM 2 that will power its Bard chatbot, among other Google products.

These recent advances in GenAI have opened a new perspective for AI in healthcare, which include simulation of medical data for model training, synthetic medical data, drug discovery, drug development, education and automation of medical notetaking, among many more applications.

AI in healthcare

The fruitful collaborative research of clinicians and AI scientists is currently leading to a growing surge in groundbreaking publications. For instance, successful use of DNNs was reported for the analysis of skin cancer images with greater accuracy than a dermatologist [8]. A deep learning system was shown effective for the diagnosis of diabetic retinopathy and related eye diseases from retinal images [9]. DNNs have also been successfully implemented in classification of ovarian cancer types [12] and cervical cancer [13] from cytological images. “Nature” have launched a new journal, *Nature Machine Intelligence*, which will provide the research community with a forum for these themes and explores a broad spectrum of topics that connect various scientific disciplines with machine intelligence.

FIGURE 18.2 Summary of AI terminology.

Transformers are among the newest and one of the most powerful classes of AI models invented to date and are regarded as “foundation models” [11].

To grasp what lies ahead requires an understanding of the breakthroughs that have enabled the rise of GenAI, which was in the making for decades. Deep learning has powered many of the recent advances in AI, but the foundation models powering GenAI applications are a step-change evolution within deep learning. Unlike previous deep learning models, they can process extremely large and varied sets of unstructured data and perform more than one task.

The revolution does not stop in academic research. The US Food and Drug Administration (FDA) has fast-tracked certain categories of AI services, opening “commercial pathways” for more than 100 AI imaging and diagnostics companies. It was recently claimed the “next big thing” in radiology may not be a new scanner technology but rather new discoveries in the way AI can be utilized for imaging.

The need for AI and the enormous potential that it holds are now clear. On one hand, the limitations of classic medicine, carried out by human caregivers, is becoming apparent—cost and human limitations are making such services more and more difficult to provide at a good standard across the entire population; on the other hand, vast improvements in computational powers and technical abilities make the incorporation of computers and machines in medical decision-making and treatment a very viable option.

In a recent paper published in The JAMA [14], the researchers evaluated the ability of ChatGPT (specifically the version that was released in November 2022) to provide quality and empathetic responses to patient questions. They used a public database of questions from a public social media forum (Reddit’s r/AskDocs) to randomly draw 195 exchanges, where a verified physician responded to a public question. Chatbot responses were generated by entering the original question into a fresh session. The original question along with anonymized and randomly ordered physician and chatbot responses were evaluated by a team of licensed health care professionals. Evaluators chose “which response was better” and judged both “the quality of information provided” and “the empathy or bedside manner provided.”

The results were in favor of the chatbot. Evaluators preferred chatbot responses to physician responses in 78.6% of the cases. Chatbot responses were of significantly higher quality than physician responses. In a post-COVID era when rapid expansion of virtual health care has caused a surge in patient messages concomitant with more work and burnout among health care professionals, this is just one example of an AI assistant that could aid in creating answers to patient questions by drafting responses that could be reviewed by clinicians.

AI for reproductive care

The idea of applying AI to infertility has been around for two decades. Early products presenting a solution which uses time-lapse imaging microscopy to collect data over the length of the embryo’s culture period, and an algorithm to predict which embryo has the best chance of progressing, are now in the market.

AI systems for IVF are already showing promising results in clinical practice. In one study, the system used human embryos to identify the ones most likely to survive. Overall, the AI system had a 67% accuracy rating [15]. This demonstrates that a fully automated model can perform better than the models based on morphokinetic parameters, and this is obtained without the need for assessment/annotation by the embryologist.

Identifying viable embryos is only the first hurdle in IVF. A true “end-to-end” AI solution for infertility care will have to integrate complex (and diverse) data sets that are currently managed in multiple, incompatible systems—patient demographics and medical histories; drug treatment regimens;

pre-implantation genetic screening; and clinical pregnancy outcome data. AI-based systems are helping physicians to choose among several treatment options that have the highest success rates, and accept new information based on the patient’s responses to treatments.

In clinical embryology, we don’t yet know the feature or set of features that is most predictive of IVF success. It is possible that the most important variable for a successful IVF cycle could still be unknown to science, but, in principle, may be uncovered by AI systems. This may be referred to as computational embryology.

A particular problem with using AI for IVF is the huge, multidimensional solution space that an AI system would have to cope with. Unlike in some other medical disciplines, there isn’t a tumour to find in a scan, an aneurysm to detect on an image, or any single feature or cluster of features that directly link to treatment success or failure. In time-lapse imaging, success or failure may be hiding in any of hundreds of images and in their relationship over time—for example, in the timing of mitosis events.

The problem is made yet more difficult by the nature of time-lapse imaging of embryos. A time-lapse video suffers from all the challenges of unsupervised photography: inconsistent lights, bad focus in part or in whole, and artefacts such as bubbles interfering with some or all frames. The embryo itself is sometimes only partly visible because it could be at the edge of the dish from the camera’s point of view. AI systems are overcoming all of these challenges and are becoming clinically useful ([Figure 18.3a–d](#)).

Biological constraints mean that only a tiny portion of this solution space represents real embryos. In AI-speak, this is a non-Euclidean problem [16] as it does not occupy a typical Euclidean space where all points are possible ([Figure 18.4a](#)). Non-Euclidean problems pose a particular challenge for neural networks, as the networks struggle to learn the relevant part of the solution space. ([Figure 18.4b–d](#) shows examples of different non-Euclidean spaces.)

Newly emerging experimental approaches in AI for non-Euclidean problems are showing great promise and it is expected that these will soon contribute to improvements in the accuracy of AI systems. In parallel, an approach to use computer vision and AI to explicitly find known features and incorporate these into the neural net is helping to mitigate the non-Euclidean nature of the problem by reducing the solution space.

AI technologies have tremendous potential to help the field of infertility medicine to transcend its current narrow focus on individual embryos and uncover new patterns hidden in the patient data for the treatment of stubborn infertility [17].

The excitement that lies ahead, albeit much further along than many have forecasted, is for software that will ingest and meaningfully process massive sets of data quickly, accurately, and inexpensively. Moreover, AI machines are predicted to see and do things that are not humanly possible. This capability will ultimately lay the foundation for high-performance medicine which is truly data-driven, negating our reliance on human resources, and eventually taking us well beyond the sum of the parts of human and machine intelligence. Reproductive medicine is likely to be one of the fields to effectively adopt the AI revolution, greatly advancing our ability to accurately prescribe personalized care for our infertility patients along with improving the success rates in the embryology lab.

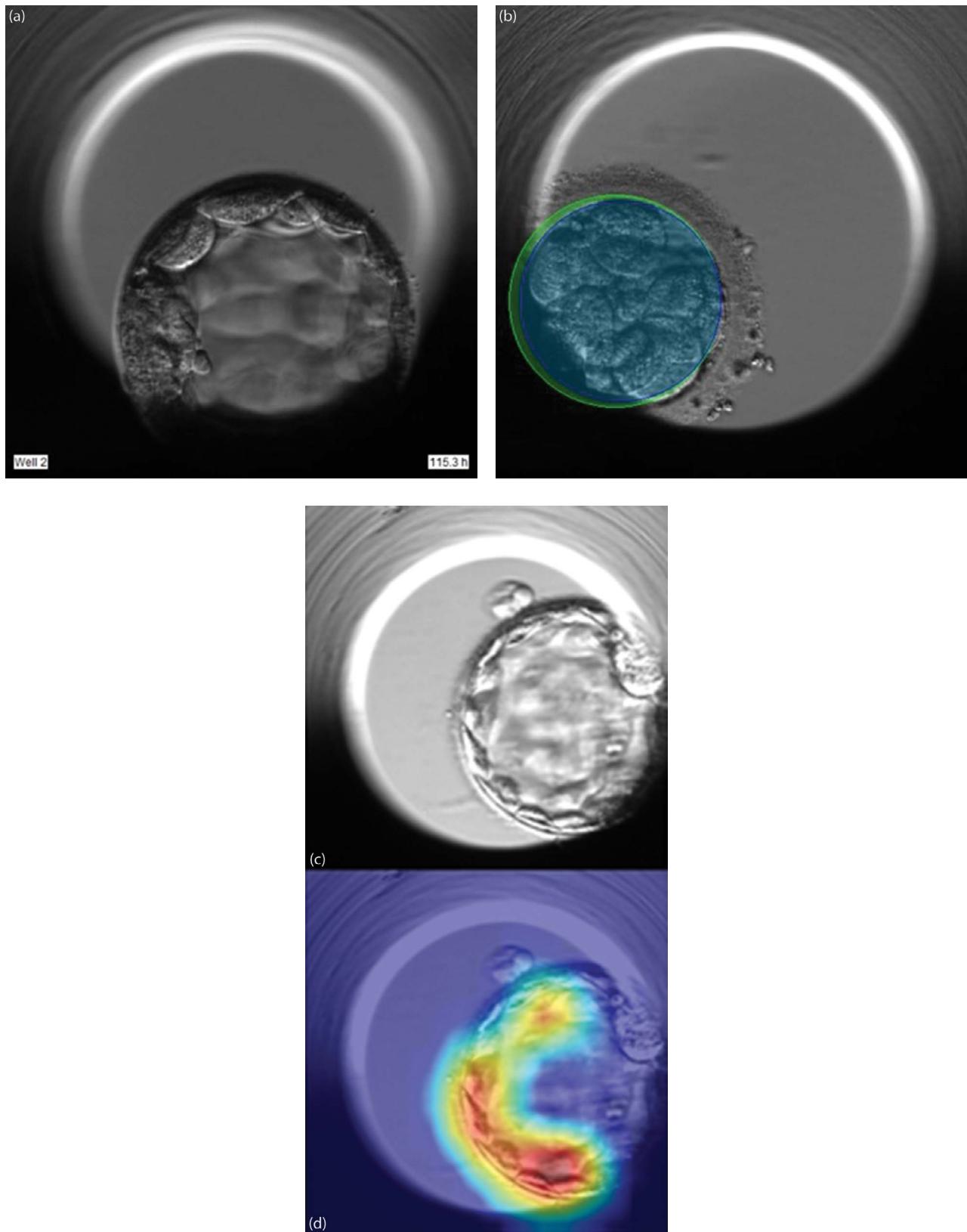


FIGURE 18.3 (a) The human brain intuitively identifies this displaced embryo at the bottom of the round dish. For scale, the dish is 200 microns in diameter. (b) Human segmentation of another displaced embryo. (c-d) Displaced embryo and the accurate detection of the model after training.

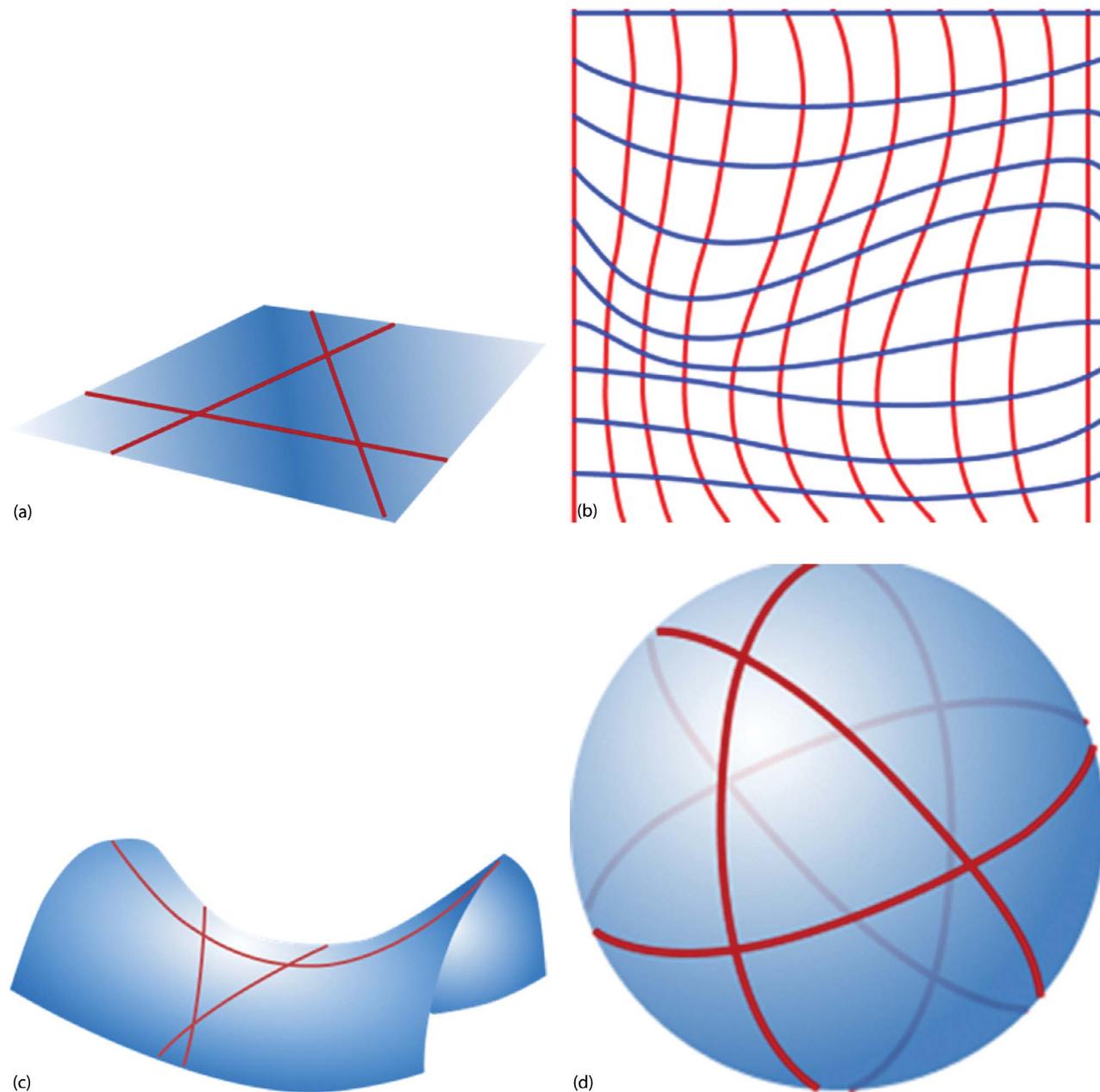


FIGURE 18.4 (a) A Euclidean space such as the physical world, where geometric rules are aligned with human intuition. (b) A two-dimension illustration of a non-Euclidean space. Coordinate lines are not parallel to each other, and each grid square does not have the same area. Mathematical problems are often represented by non-Euclidean spaces, but to our intuition these spaces seem “wrong.” (c) Non-Euclidean space-hyperbolic. The angles of a triangle add up to more than 180 degrees, as opposed to a triangle in a Euclidean space. (d) Non-Euclidean space-spherical. The angles of a triangle add up to less than 180 degrees.

References

1. Editorial. Artificial intelligence in health care: Within touching distance. *Lancet*. 2017;390(10114):2739.
2. Rudin P. Thoughts on human learning vs. machine learning, Essay. January 2017. <https://singularity2030.ch/thoughts-on-human-learning-vs-machine-learning/>
3. Ronaghan S. Deep Learning: Overview of neurons and activation functions. Medium. July 2018. <https://medium.com/@srnghn/deep-learning-overview-of-neurons-and-activation-functions-1d98286cf1e4>
4. Leijnen S, van Veen F. The neural network zoo. Multidisciplinary Digital Publishing Institute Proceedings. 2020;47:1:9, <https://www.asimovinstitute.org/neural-network-zoo>
5. Turing AM. On computable numbers with an application to the Entscheidungs problem. *Proc Lond Math Soc*. 1936;s2-42: 230–265.
6. Turing AM. Computing machinery and intelligence. *Mind*. 1950;59:433–460.
7. McCulloch WS, Pitts W. A logical calculus of the ideas immanent in nervous activity. *Bull Math Biophys*. 1943;5:115–133.

8. Yu C, Yang S, Kim W, Jung J, Chung KY, Lee SW, Oh B. Correction: Acral melanoma detection using a convolutional neural network for dermoscopy images. PLOS ONE. 2018;13(4):e0196621.
9. Wei Ting DS. Development and validation of a deep learning system for diabetic retinopathy and related eye diseases using retinal images from multiethnic populations with diabetes. JAMA. 2017;318:2211–23.
10. Vaswani A, Shazeer N, Parmar N, Uszkoreit J, Jones L, Gomez AN, Kaiser L, Polosukhin I. Attention is all you need. 2023. arXiv:1706.03762.
11. Bommasani R, et al. On the opportunities and risks of foundation models. 2022. arXiv:2108.07258.
12. Raza SEA, AbdulJabbar K, Jamal-Hanjani M, Veeriah S, Le Quesne J, Swanton C, Yinyin Yuan Y. Deconvolving convolution neural network for cell detection. 2018;arXiv:1806.06970v1.
13. Hu L, Bell D, Antani S, Xue Z, Yu K, Horning MP, Gachuhi N, Wilson B, Jaiswal MS, Befano B, Long LR, Herrero R, Einstein MH, Burk RD, Demarco M, Gage JC, Rodriguez AC, Wentzensen N, Schiffman M. An observational study of deep learning and automated evaluation of cervical images for cancer screening. J Natl Cancer Inst. 2019;111(9):923–32. <https://doi.org/10.1093/jnci/djy225>.
14. Ayers JW, Dredze M, Leas EC, Zhu Z, Kelley JB, Faix DJ, Goodman AM, Longhurst CA, Hogarth M, Smith DM. Comparing Physician and Artificial Intelligence Chatbot Responses to Patient Questions Posted to a Public Social Media Forum. JAMA Internal Medicine. 2023.
15. Berntsen J, Rimestad J, Lassen JT, Tran D, Kragh MF, Robust and generalizable embryo selection based on artificial intelligence and time-lapse image sequences. PLOS ONE. 2022;17(2):e0262661. <https://doi.org/10.1371/journal.pone.0262661>
16. Bronstein MM, Bruna J, LeCun Y, Szlam A, Vandergheynst P. Geometric deep learning: going beyond Euclidean data. 2017;arXiv:1611.08097v2 [cs.CV].
17. Tzukerman N, Rotem O, Shapiro MT, Maor R, Meseguer M, Gilboa D, Seidman DS, Zaritsky A. Using Unlabeled Information of Embryo Siblings from the Same Cohort Cycle to Enhance In Vitro Fertilization Implantation Prediction. Adv Sci (Weinh). 2023 Jul 28:e2207711

19

ARTIFICIAL INTELLIGENCE (AI) IN GAMETE AND EMBRYO SELECTION

Chloe He, Neringa Karpaviciute, Cristina Hickman, and Nikica Zaninovic

Over the past few decades, computers have revolutionized the field of medicine. Today, computers and computer-based technologies have become an indispensable tool for clinicians, researchers, and patients, supporting their efforts to monitor conditions, process data, and access healthcare services. But the field of computing itself is also witnessing its own revolution. Since the early 2010s, the collection of large data sets and increases in computing power have enabled numerous successes in the field of artificial intelligence (AI). Many of these successes have hinged on machine learning (ML), a data-driven subfield of AI that focuses on the automatic discovery of patterns in data.

The plethora of techniques coming out of AI research have the potential to resolve many of the challenges experienced within the field of reproductive medicine, including prediction, classification, and operational efficiency. If incorporated effectively into clinical processes, the automation associated with AI could enhance consistency, efficiency, and efficacy as well as reduce risk and human errors. The promise of AI has led to an increase in research towards the development of AI-based tools to assist in gamete and embryo selection. In this chapter, we will review the current landscape of AI in gamete and embryo selection in the *in vitro* fertilization (IVF) laboratory.

AI in gamete selection

Ensuring gamete quality is a crucial step towards assisted reproductive technology (ART) cycle success. Manual gamete selection is a labour-intensive, time-consuming, and highly subjective process. The application of AI to oocyte and sperm selection greatly benefits gamete donation and cryopreservation, and it reduces embryo waste, particularly in countries with restrictive ART laws. AI-based oocyte selection has the potential to be able to identify oocytes with the highest developmental potential that are “worth” fertilizing. This could increase the efficiency of the IVF process and reduce the creation of incompetent, nonviable, or chromosomally abnormal embryos that will never be used in clinical treatment. It could also reduce storage requirements for cryopreserved oocytes and embryos. The following sections explore gamete selection methods driven by ML. A summary of the studies discussed can be found in [Table 19.1](#).

Oocyte selection

The ability to noninvasively assess oocyte quality without damaging the oocyte itself is key to the success of ART cycles. Such assessment typically involves visual inspection of the cumulus–oocyte complex, cytoplasm, zona pellucida, perivitelline space, and polar bodies. Because the appearance of these structures has been associated with successful fertilization, ML algorithms have been aimed at analysing them [\[1\]](#).

Early attempts used classical image analysis methods to predict oocyte capacity to result in live birth [\[2, 3\]](#). Manna et al. [\[4\]](#) used multiple artificial neural networks to predict pregnancy from oocyte images using textural descriptors of the oocytes

(derived local binary patterns). The textural descriptors were obtained using classical image analysis techniques and were fed into each network. A classification was arrived at through a majority-voting procedure between the networks. More recently, Baručić et al. [\[5\]](#) proposed a system for the automatic detection of oocytes with high developmental potential. Unlike Manna et al. [\[4\]](#), Baručić et al. [\[5\]](#) considered physical measurements. Individual parts of the oocyte were segmented (that is, identified and highlighted) using a convolutional neural network (CNN) that took the raw oocyte images as input and returned segmentation maps. Measurements derived from the segmentation maps were then fed into a support vector machine model that predicted the oocyte’s viability. The model achieved performance comparable to that of a human embryologist.

Several studies have also solely focused on the automatic identification and segmentation of key oocyte structures such as vacuoles and polar bodies. The systems developed in these studies have several applications, including downstream image analysis [\[5\]](#) and robotic cell manipulation [\[6\]](#). Firuzinia et al. [\[7\]](#) developed a deep learning system for segmenting low-resolution images of metaphase II (MII) oocytes. Targosz et al. [\[8\]](#), carried out a benchmark study of popular CNN architectures for the segmentation of cytoplasm, vacuoles, polar bodies, zona pellucida, cumulus cells, and other structures.

Semen analysis and sperm selection

Although a limited number of oocytes are retrieved for an IVF cycle, tens of thousands of sperm are placed around an oocyte during IVF. Sperm sorting must take place in the embryology laboratory to select sperm with high fertilization potential. Research into the application of AI systems to sperm evaluation, sorting, and selection has generally been more advanced than that for oocytes, with many major laboratories making use of computer-assisted sperm assessment systems. Sperm morphology, motility, and concentration are important parameters evaluated in semen analyses by embryologists and andrologists. Moreover, unlike oocytes, visually identifiable parameters such as sperm motility and morphology have been shown to reflect DNA integrity [\[9\]](#). As a result, AI systems have been developed to automate this evaluation to save time and avoid the subjectivity and variability inherent in manual assessment.

Several studies have proposed automated systems for sperm morphology analysis. Among the earliest of these systems is FERTECH, which classified sperm according to World Health Organization (WHO) criteria [\[10\]](#). More recent studies have tackled the problem using modern data-driven image analysis techniques [\[11–21\]](#). While most of these works analysed the spermatozoon as a whole, Javadi et al. [\[12\]](#) developed a CNN-based system that explicitly separated the evaluation of the head, vacuole, and acrosome of the sperm.

The assessment of sperm motility has also been a target for automation [\[22–27\]](#). Goodson et al. [\[24\]](#) developed a support vector machine model that reported motility characteristics

TABLE 19.1 AI Gamete Selection Literature

Gamete	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
Oocyte	Image Segmentation	Basile et al. (2010)	[2]	A texture-based image processing approach for the description of human oocyte cytoplasm	Identified and segmented the cytoplasm in oocyte images. Created clusterings of cytoplasm textures.	No
		Targosz et al. (2021)	[8]	Semantic segmentation of human oocyte images using deep neural networks	Benchmarked different convolutional neural network (CNN) architectures for segmentation of key structures in images of oocytes.	No
		Firuzinia et al. (2021)	[7]	A robust deep learning-based multiclass segmentation method for analyzing human metaphase II oocyte images	Proposed a CNN model for key structures in human metaphase II oocytes.	No
Developmental Potential Prediction	Developmental Potential Prediction	Manna et al. (2013)	[4]	Artificial Intelligence (AI) techniques for embryo and oocyte classification	Predicted live birth from textural descriptors of oocytes using an ensemble of Levenberg-Marquardt neural networks.	No
		Baručić et al. (2021)	[5]	Automatic evaluation of human oocyte developmental potential from microscopy images	Extracted measurements from CNN-generated oocyte segmentation maps and used them to predict developmental potential.	Yes
Sperm	Morphological Analysis	Kruger et al. (1993)	[10]	A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading.	Proposed an image analysis program for the classification of sperm morphology according to strict criteria.	Yes
		Shaker et al. (2017)	[15]	A dictionary learning approach for human sperm heads classification	Used an adaptive patch-based dictionary learning method to classify sperm heads. Publicly released the widely used HuSHeM data set of sperm heads images.	No
		Riordon et al. (2019)	[14]	Deep learning for the classification of human sperm	Fine-tuned an existing VGG16 CNN to classify sperm head morphology from images.	No
		Javadi and Mirroshandel (2019)	[12]	A novel deep learning method for automatic assessment of human sperm images	Proposed a CNN with low computational cost for the assessment of morphological deformities in head, acrosome, neck, tail, and vacuole.	No
		Ilhan et al. (2020)	[17]	A fully automated hybrid human sperm detection and classification system based on mobile-net and the performance comparison with conventional methods	Proposed a system for the segmentation and classification of sperm images.	No
		Yüzkat et al. (2021)	[16]	Multi-model CNN fusion for sperm morphology analysis	Trained six CNNs on different data sets to assess sperm morphology. At inference time, predictions from all models are combined via a voting procedure to arrive at a final assessment.	No
		Abbasi et al. (2021)	[18]	Effect of deep transfer and multi-task learning on sperm abnormality detection	Adapted an existing VGG19 CNN to detect head, acrosome, and vacuole abnormalities in sperm images.	No
		Sato et al. (2022)	[26]	A new deep-learning model using YOLOv3 to support sperm selection during intracytoplasmic sperm injection procedure	Trained a YOLOv3 CNN to simultaneously track and morphologically assess sperm in real time.	No
		Chandra et al. (2022)	[19]	Prolificacy assessment of spermatozoan via state-of-the-art deep learning frameworks	Benchmarked different CNN architectures for the detection of head, acrosome, and vacuole abnormalities.	Yes

(Continued)

TABLE 19.1 AI Gamete Selection Literature (Continued)

Gamete	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
Motility Analysis	Goodson et al. (2017)	[24]	CASA nova: A multiclass support vector machine model for the classification of human sperm motility patterns	Analysed kinematic parameters of sperm obtained through sperm tracking software using a support vector machine to assess motility.	No	
	Dimitriadiis et al. (2019)	[31]	Automated smartphone-based system for measuring sperm viability, DNA fragmentation, and hyaluronic binding assay score	Proposed a smartphone-based video analysis system for the assessment of sperm viability using 3D-printed hardware.	No	
	Agarwal et al. (2019)	[27]	Automation of human semen analysis using a novel AI optical microscopic technology	Comparison between a proprietary automated semen analysis device and human experts on assessment of sperm concentration and motility.	Yes	
	Hicks et al. (2019)	[28]	Machine learning (ML)-based analysis of sperm videos and participant data for male fertility prediction	Trained a CNN to predict sperm motility from multimodal video data and clinical data.	No	
	Somasundaram et al. (2021)	[29]	Faster region CNN and semen tracking algorithm for sperm analysis	Combined the outputs of a faster region CNN network with other algorithms for the assessment of sperm morphology and motility from video data.	No	
	Alameri et al. (2021)	[23]	Multistage optimization using a modified Gaussian mixture model in sperm motility tracking	Presented a system for the evaluation of motility from videos based on a modified Gaussian mixture model.	No	
	Valiuškaitė et al. (2021)	[22]	Deep learning based evaluation of spermatozoid motility for artificial insemination	Trained a faster region CNN network for the assessment of sperm morphology and motility from videos.	No	
	Chang et al. (2014)	[11]	Gold-standard and improved framework for sperm head segmentation	Used classical image analysis techniques to perform segmentation of the sperm head, nucleus, and acrosome.	No	
Image Segmentation	Movahed et al. (2019)	[20]	Automatic segmentation of sperm's parts in microscopic images of human semen smears using concatenated learning approaches	Proposed a system proposed of a variety of ML algorithms (including CNNs, k-means clustering, and support vector machines) for the segmentation of the head, acrosome, nucleus, axial filament, mid-piece, and tail.	No	
	Kandel et al. (2020)	[13]	Reproductive outcomes predicted by phase imaging with computational specificity of spermatozoon ultrastructure	Proposed a U-Net CNN for the segmentation of the head, mid-piece, and tail from high-sensitive phase imaging data. These segmentations were used to calculate dry-mass ratios between the different parts of the sperm.	No	
	Marín et al. (2021)	[21]	Impact of transfer learning for human sperm segmentation using deep learning	Demonstrated that a CNN pre-trained on larger sperm data set for segmentation can be adapted to new sperm data sets via transfer learning leading to performance gains over training from scratch.	No	

(Continued)

TABLE 19.1 AI Gamete Selection Literature (*Continued*)

Gamete	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
DNA Integrity Prediction	McCallum et al. (2019)	[32]	Deep learning-based selection of human sperm with high DNA integrity	Fine-tuned an existing VGG16 CNN to predict DNA integrity from brightfield images.	No	
	Zhang et al. (2021)	[9]	Quantitative selection of single human sperm with high DNA integrity for intracytoplasmic sperm injection	Created a program to predict DNA fragmentation from morphological and motility assessments derived from computer vision algorithms.	Yes	
Sperm Ranking	Mendizabal-Ruiz et al. (2022)	[25]	Computer software (SiD) assisted real-time single sperm selection correlates with fertilization and blastocyst formation	Evaluated a proprietary sperm scoring system with respect to whether human embryologists selected sperm for ICSI, fertilization, and blastocyst formation.	No	
Clinical Outcome Prediction	Gunderson et al. (2021)	[30]	Machine-learning algorithm incorporating capacitated sperm intracellular pH predicts conventional in vitro fertilization success in normospermic patients	Predicted fertilization after conventional IVF using gradient-boosted ML algorithm trained on clinical data, sperm pH, and membrane potential.	No	

with 89.9% accuracy. The model was also able to identify subpopulations of sperm cells [24]. More recently, deep learning methods have been used to improve the accuracy achieved by computer-assisted motility analysis [22, 28, 29]. Mendizabal-Ruiz et al. [25] used a proprietary computer vision system to select individual spermatozoon for intracytoplasmic sperm injection (ICSI) based on kinematic data (straight-line velocity, linearity of the curvilinear path, and head movement pattern). They found statistically significant differences between spermatozoa that resulted in IVF success and those that did not. Furthermore, Sato et al. [26] developed a CNN-based system to simultaneously perform morphological assessment and tracking of sperm in real time to assist in sperm selection for ICSI. The model was able to distinguish between normal and abnormal sperm with high sensitivity and precision (79.4% and 88.1%, respectively).

Other sperm selection techniques include measuring intracellular sperm pH, which was found to correlate with IVF success [30]. Furthermore, smartphone-based semen analysis systems are becoming more popular, which enable at-home testing [31]. A study by McCallum et al. [32] was the first to demonstrate that it is possible to predict the DNA integrity of an individual spermatozoon solely from imaging data by training a CNN with sperm images labelled with different DNA fragmentation indices.

AI in embryo selection

The application of AI to embryo selection in ART cycles has also been an area of considerable research interest. Most of these approaches are based on the analysis of both static and time-lapse embryo imaging data. As a result, many state-of-the-art systems have been based on deep learning, a subset of AI approaches that have proved especially successful at image analysis tasks. In this section, we will take a high-level look at the landscape for AI systems for embryo selection. A summary of the studies discussed can be found in Table 19.2.

What is the ground truth?

To date, almost all work in AI for embryo selection has involved the use of supervised learning to predict a clinically relevant outcome from input data. While the input data is usually time-lapse or static imagery, there is a wide range of target variables in the literature that include blastocyst formation [33–35], blastocyst grade [36–39], ploidy [40–44], implantation [33, 40, 45–50], fetal heartbeat pregnancy [47, 48, 51–57], and live birth [52, 57–61].

This heterogeneity arises from several factors. Clearly, the different outcomes carry different biological and clinical significance. For instance, it may be argued that automated embryo selection systems should be trained using live birth data since a live birth is the end goal of the ART cycle. It may, however, also be argued that the occurrence of live birth greatly depends on factors other than the embryo, and thus the prediction of blastocyst grade or implantation may be more appropriate. Another factor is data availability and volume; as a rule of thumb, the more developmentally advanced an embryo must be to measure a target variable, the scarcer the data available on the target variable will be by virtue of fewer embryos reaching that stage. A more comprehensive treatment of the advantages and disadvantages of each prediction target can be found in Table 19.3.

Static image analysis

Many early studies examining the potential for the use of AI in embryo selection focused on single static images. Khosravi et al. [37] fine-tuned an InceptionV3 CNN model to classify Hoffman modulation contrast (HMC) microscopy images of D5 blastocysts as “good” or “poor.” The ground-truth classifications were based on the majority vote of a panel of four embryologists. The model outperformed each individual embryologist, predicting the majority vote with 96.9% accuracy. Ver Milyea et al. [55] also made use of a CNN. In this study, it was to predict fetal heartbeat from D5 blastocyst images captured on a standard light microscope. The system achieved 64.3% accuracy with 70.1% sensitivity and 60.5% specificity.

TABLE 19.2 AI Embryo Selection Literature

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
Static Image	Embryo Grading	Filho et al. (2012)	[36]	A method for semi-automatic grading of human blastocyst microscope images	Used measurements derived from segmentation maps of blastocysts to train a support vector machine to predict blastocyst grade.	No
		Khosravi et al. (2019)	[37]	Deep learning enables robust assessment and selection of human blastocysts after in vitro fertilization	Fine-tuned an existing InceptionV3 CNN to predict blastocyst grade. The system outperformed individual embryologists at embryo selection.	Yes
		Wu et al. (2020)	[62]	A classification system of day 3 human embryos using deep learning	Proposed an ensemble of CNN models for the prediction of gradings for D3 embryos.	Yes
		Thirumalaraju et al. (2021)	[39]	Evaluation of deep CNNs in classifying human embryo images based on their morphological quality	Benchmarked several CNN architectures for the prediction of developmental stage and, where applicable, blastocyst grade of D5 embryos. Also generated heatmaps to help identify parts of the image deemed to be important by the models.	No
Image Enhancement	Image Segmentation	Raudonis et al. (2021)	[73]	Fast multi-focus fusion based on deep learning for early-stage embryo image enhancement	Proposed a CNN-based system to fuse multiple focal planes into a single image without the loss of useful information.	No
		Singh et al. (2015)	[68]	Automatic segmentation of trophectoderm in microscopic images of human blastocysts	Used a level-set algorithm to segment the trophectoderm in blastocyst images.	No
		Saeedi et al. (2017)	[70]	Automatic identification of human blastocyst components via texture	Used a pipeline of classical computer vision techniques to segment the trophectoderm and inner cell mass.	No
		Rad et al. (2019)	[67]	BLAST-NET: Semantic segmentation of human blastocyst components via cascaded atrous pyramid and dense progressive upsampling	Proposed the first CNN architecture for the segmentation of the zona pellucida, trophectoderm, inner cell mass, and blastocoel.	No
		Rad et al. (2020)	[66]	Trophectoderm segmentation in human embryo images via inceptioned U-Net	Proposed a novel U-Net CNN architecture for the segmentation of the zona pellucida, trophectoderm, and inner cell mass.	No
		Arsalan et al. (2022)	[71]	Detecting blastocyst components by AI for human embryological analysis to improve success rate of in vitro fertilization	Proposed a novel CNN architecture using sprint convolutional blocks for the segmentation of the zona pellucida, trophectoderm, inner cell mass, and blastocoel.	No
Live Birth Prediction	Manna et al. (2013)	[4]		AI techniques for embryo and oocyte classification	Predicted live birth from textural descriptors of embryos using an ensemble of Levenberg-Marquard neural networks.	No

(Continued)

TABLE 19.2 AI Embryo Selection Literature (*Continued*)

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
		Geller et al. (2021)	[48]	An AI-based algorithm for predicting pregnancy success using static images captured by optical light microscopy during intracytoplasmic sperm injection	Finetuned an existing InceptionV1 CNN to predict pregnancy and live birth from pictures of D5 embryos.	No
		B. Huang et al. (2022)	[52]	Using deep learning to predict the outcome of live birth from more than 10,000 embryo data	Proposed a CNN model for the prediction of live birth outcome from a single blastocyst transfer using imaging data.	No
		Enatsu et al. (2022)	[47]	A novel system based on AI for predicting blastocyst viability and visualizing the explanation	Proposed a CNN model for the prediction of pregnancy and live birth from images of D5 embryos. In addition, explanations for the CNN's decisions were provided using heatmaps. The model outperformed embryologist evaluation using the Gardner scale.	Yes
Pregnancy Prediction	Bormann et al. (2020)		[45]	Performance of a deep learning based neural network in the selection of human blastocysts for implantation	Evaluated a system for the automatic prediction of implantation against fifteen trained embryologists. The system was based on a CNN combined with genetic algorithms. The system outperformed the embryologists.	Yes
			[46]	Predicting pregnancy test results after embryo transfer by image feature extraction and analysis using ML	Compared several algorithms for the prediction of biochemical pregnancy from parameters derived from dimensionality reduction and image analysis of D5 and D6 blastocysts.	No
			[55]	Development of an AI-based assessment model for prediction of embryo viability using static images captured by optical light microscopy during IVF	Presented a CNN model for the prediction of pregnancy from images of D5 blastocysts. The model significantly outperformed embryologists.	Yes
	Fitz et al. (2021)		[49]	Should there be an "AI" in TEAM? Embryologists' selection of high implantation potential embryos improves with the aid of an AI algorithm	Demonstrated improvements in the ability of embryologists to select embryos with high implantation potential when aided by a CNN-based system.	Yes
	Loewke et al. (2022)		[56]	Characterization of an AI model for ranking static images of blastocyst stage embryos	Proposed a CNN model for the prediction of pregnancy from images of blastocysts. Explanations for the CNN's decisions were provided using heatmaps. Inspection of the heatmaps revealed the features learned by the model overlapped with the features considered by manual grading systems.	Yes

(Continued)

TABLE 19.2 AI Embryo Selection Literature (Continued)

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
	Ploidy Prediction	Chavez-Badiola (2020)	[40]	embryo ranking intelligent classification algorithm (ERICA): AI clinical assistant predicting embryo ploidy and implantation	Trained a deep neural network to predict ploidy and implantation from parameters derived from the analysis of blastocyst images. The network outperformed two human embryologists.	Yes
	Image Generation	Dirvanauskas et al. (2019)	[87]	HEMIGEN: Human embryo image generator based on generative adversarial networks	Trained a generative adversarial network to generate synthetic images of cleavage stage embryos.	No
	Pronuclei Detection	Fukunaga et al. (2020)	[75]	Development of an automated two pronuclei detection system on time-lapse embryo images using deep learning techniques	Presented a CNN model for the detection of pronuclei in fertilized oocytes.	No
Time-lapse	Embryo Grading	Kragh et al. (2019)	[38]	Automatic grading of human blastocysts from time-lapse imaging	Presented a recurrent CNN-based system for the prediction of inner cell mass and trophectoderm grades from time-lapses. The model performed on par with embryologists.	Yes
	Pregnancy Prediction	Tran et al. (2019)	[54]	Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer	Introduced a deep learning model for the prediction of fetal heart beat pregnancy from time-lapse videos.	No
		Alegre et al. (2021)	[50]	Assessment of embryo implantation potential with a cloud-based automatic software	Predicted implantation potential using morphokinetic parameters obtained by a CNN model.	No
		Kan-Tor et al. (2021)	[33]	Automated evaluation of human embryo blastulation and implantation potential using deep-learning	Predicted blastocyst formation and implantation using a system based on deep neural networks. To gain some insight into the features the system deemed most important, the trained models were analysed using Shapley additive explanations.	No
		Berntsen et al. (2022)	[51]	Robust and generalizable embryo selection based on AI and time-lapse image sequences	Proposed a deep learning model for the prediction of fetal heart beat pregnancy from time-lapses. The model consisted of a two-stream inflated 3D CNN augmented with bidirectional long short-term memory modules.	No
		Kragh et al. (2022)	[53]	Predicting embryo viability based on self-supervised alignment of time-lapse videos	Proposed a self-supervised training set-up that enabled the use of unlabelled data in the training of deep learning models. Models trained to predict pregnancy using the proposed method outperformed models trained with supervised learning alone.	No

(Continued)

TABLE 19.2 AI Embryo Selection Literature (*Continued*)

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
	Erlich et al. (2022)	[82]	Pseudo contrastive labeling for predicting IVF embryo developmental potential	Proposed a pseudo-contrastive labelling scheme that assigned similar labels to embryos with similar developmental patterns. This allowed a CNN-based system to be trained to predict implantation despite ambiguity arising from multiple embryo transfers and missing labels. The model outperformed human embryologists.	Yes	
Live Birth Prediction	Sawada et al. (2021)	[61]	Evaluation of AI using time-lapse images of IVF embryos to predict live birth	Trained an attention branch network to predict live birth from time-lapse videos.	No	
	Nagaya et al. (2022)	[60]	Embryo grading with unreliable labels due to chromosome abnormalities by regularized PU learning with ranking	Presented a new training scheme for deep learning models for the prediction of live birth in the presence of confounders such as chromosome abnormalities.	No	
Ploidy Prediction	B. Huang et al. (2021)	[43]	An AI model (euploid prediction algorithm) can predict embryo ploidy status based on time-lapse data	Presented a pipeline of deep learning models trained to predict ploidy from time-lapse videos.	No	
	Lee et al. (2021)	[44]	End-to-end deep learning for recognition of ploidy status using time-lapse videos	Trained a two-stream inflated 3D CNN for the prediction of ploidy from time-lapse videos.	No	
Image Segmentation	Zhao et al. (2021)	[69]	Application of CNN on early human embryo segmentation during in vitro fertilization	Proposed a CNN model for the segmentation of the cytoplasm, pronuclei, and zona pellucida in D1 embryo time-lapses.	No	
	T. T. F. Huang et al. (2021)	[80]	Deep learning neural network analysis of human blastocyst expansion from time-lapse image files	Trained a U-Net CNN model to segment blastocysts. The model was applied to time-lapses and blastocyst expansion curves were generated.	No	
Blastocyst Formation Prediction	Coticchio et al. (2021)	[81]	Cytoplasmic movements of the early human embryo: imaging and AI to predict blastocyst development	Evaluated several ML models for the prediction of blastocyst formation from the movement of cytoplasmic particles. Movement of the particles was measured using a particle image velocimetry algorithm.	No	
	Liao et al. (2021)	[34]	Development of deep learning algorithms for predicting blastocyst formation and quality by time-lapse monitoring	Trained a long short-term memory neural network to predict blastocyst formation and quality from morphokinetic parameters derived from a CNN model.	Yes	
Developmental Stage Prediction	Lau et al. (2019)	[76]	Embryo staging with weakly-supervised region selection and dynamically-decoded predictions	Predicted embryo developmental stage using a CNN model augmented with monotonicity constraints. Images were automatically cropped by a reinforcement learning agent as a pre-processing step.	No	

(Continued)

TABLE 19.2 AI Embryo Selection Literature (Continued)

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
Morphokinetic Annotations	Leahy et al. (2020)	[65]	Automated Measurements of Key Morphological Features of Human Embryos for IVF	Predicted embryo developmental stage and cell segmentation masks using a CNN-based system.	No	
	Malmsten et al. (2020)	[79]	Automated cell division classification in early mouse and human embryos using CNNs	Trained a CNN to detect and classify cell divisions up to the 8-cell stage.	No	
	Lockhart et al. (2021)	[78]	Automating embryo development stage detection in time-lapse imaging with synergic loss and temporal learning	Proposed a novel CNN model and loss function for the detection of developmental stages in time-lapses.	No	
	Lukyanenko et al. (2021)	[77]	Developmental stage classification of embryos using two-stream neural network with linear-chain conditional random field	Proposed a novel system for the detection of developmental stages in time-lapses. The system consisted of two CNNs (one for the prediction of developmental stage and the other for the detection of transitions between stages) and a linear-chain conditional random field.	No	
Morphokinetic Annotations	Pregnancy Prediction	Petersen et al. (2016)	[74]	Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on day 3	Predicted pregnancy from manually provided morphokinetic annotations using a decision tree approach.	No
Clinical Data	Live Birth Prediction	Bodri et al. (2018)	[59]	Predicting live birth by combining cleavage and blastocyst-stage time-lapse variables using a hierarchical and a data mining-based statistical model	Trained two models for the prediction of live birth from morphokinetic parameters. The first was a hierarchical model. The second was a logistic regression model using features derived from principal component analysis.	No
	D'Estaing et al. (2021)	[35]	An ML system with reinforcement capacity for predicting the fate of an ART embryo	Predicted blastocyst formation and live birth using a scoring system based on logistic regression over morphokinetic parameters.	No	
Ploidy Prediction	de Gheselle et al. (2022)	[42]	ML for prediction of euploidy in human embryos: In search of the best-performing model and predictive features	Benchmarked several ML models for the prediction of euploidy from clinical and morphokinetic data.	No	
Clinical Data	Live Birth Prediction	Amini et al. (2021)	[58]	Factors associated with in vitro fertilization live birth outcome: A comparison of different classification methods	Benchmarked several ML models for the prediction of live birth from demographic, clinical, and treatment parameters.	No

(Continued)

TABLE 19.2 AI Embryo Selection Literature (*Continued*)

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
Pregnancy Prediction	Liu et al. (2021)	[57]	Multifactor prediction of embryo transfer outcomes based on a ML algorithm	Benchmarked several ML models for the prediction of pregnancy following frozen embryo transfer from demographic, clinical and treatment parameters.	No	
Optimal Transfer Policy Prediction	Xi et al. (2021)	[63]	Individualized embryo selection strategy developed by stacking ML model for better in vitro fertilization outcomes: An application study	Combined patient factors with embryologist gradings of D3 embryos to propose a personalized embryo transfer strategy that maximized chances of pregnancy within a given risk of twin pregnancy.	No	
Proteomic Profile	Live Birth Prediction	Bori et al. (2021)	[64]	An AI model based on the proteomic profile of euploid embryos and blastocyst morphology: A preliminary study	Trained a multilayer perceptron for the prediction of live birth based on blastocyst morphology and the protein profiles of spent culture media.	No
Chromosome Sequencing	Pregnancy Prediction	Chen et al. (2022)	[41]	Non-invasive embryo selection strategy for clinical IVF to avoid wastage of potentially competent embryos	Predicted blastocyst ploidy from chromosome sequencing of the culture medium using a range of ML models. The best model (a random forest) was validated in a blinded prospective observational study.	No

TABLE 19.3 Comparison of Common Prediction Targets for Embryo Selection AI

Target	For	Against
Blastocyst Formation	<ul style="list-style-type: none"> • Lots of data available • Objective 	<ul style="list-style-type: none"> • Not a strong predictor of pregnancy or live birth
Blastocyst Grade	<ul style="list-style-type: none"> • Lots of data available • Correlation with pregnancy and live birth 	<ul style="list-style-type: none"> • Requires embryologist annotation • Subjective, with variation between annotators
Ploidy	<ul style="list-style-type: none"> • Correlation with early pregnancy loss 	<ul style="list-style-type: none"> • Requires biopsy • Relatively little data available
Implantation (Biochemical Pregnancy)	<ul style="list-style-type: none"> • Strong correlation with live birth 	<ul style="list-style-type: none"> • Little data available • Multiple embryo transfers can make it difficult to attribute implantation to a single embryo
Fetal Heartbeat	<ul style="list-style-type: none"> • Strong correlation with live birth 	<ul style="list-style-type: none"> • Little data available • Maternal factors can have a confounding effect • Multiple embryo transfers can make it difficult to attribute the pregnancy to a single embryo
Live Birth	<ul style="list-style-type: none"> • Corresponds to the ultimate goal of the ART cycle 	<ul style="list-style-type: none"> • Little data available • Maternal and environmental factors can have a confounding effect • Multiple embryo transfers can make it difficult to attribute the birth to a single embryo

Automated static image analysis has also been applied by Wu et al. [62] to classify D3 cleavage-stage embryos. Their classification scale consisted of four categories and captured the equality in blastomere size as well as the severity of fragmentation. The CNN-based system achieved 74.1% accuracy and an area under the curve (AUC) of 0.935. Other recent work has looked at combining insights from static image analysis with other data. For instance, Xi et al. [63] combined patient factors with embryologist gradings of D3 embryos to propose a personalized embryo transfer strategy (single/double embryo transfer along with specific embryos to transfer) that maximized chances of pregnancy within a given risk of twin pregnancy. The system used the XGBoost algorithm and achieved an AUC of 0.72 for the prediction of twin pregnancy. In a preliminary study, Bori et al. [64] used a multilayer perceptron to analyse how protein profiles of spent culture media and blastocyst morphology contribute to live birth. The system was able to predict live birth with 72.7% accuracy.

Not all research in static image analysis, however, has focused on classifying embryo images according to clinical outcomes. Several studies have proposed systems for image segmentation [65–71]. One such system proposed by Rad et al. [67] used a CNN to identify the zona pellucida, trophectoderm, inner cell mass, and blastocoel in images of blastocysts. Once segmented, each structure can be analysed separately in downstream tasks such as taking measurements or assigning quality grades. Other work has focused on pre-processing and enhancing images before they are used as input into models [72, 73].

Time-lapse analysis

The majority of recent studies on AI for embryo selection focus on the analysis of time-lapse videos captured on incubators such as the EmbryoScope, Geri, and MIRI. An advantage of using these incubation systems is that they allow a high degree of standardization in imaging set-ups between clinics. Early systems utilizing time-lapse analysis relied on the manual annotation of morphokinetic events by embryologists. An example of these systems (that is, both commercially available and FDA-approved) is the Known implantation data on day 3 (KIDSscoreD3) [74], a decision tree algorithm trained on 3275 embryos with known outcome data that predicted blastocyst formation and blastocyst quality from morphokinetic markers during the cleavage stage.

Since around 2020, the focus has shifted towards the use of fully automated image analysis. A clear advantage of taking such an approach is that it avoids intra- and inter-operator variation in embryologist evaluation and saves time from manual annotation. As with static image analysis, most of these systems are based on CNNs (albeit slightly modified using techniques such as long short-term memory modules to handle the temporal aspect of the data).

Broadly speaking, most research into automated time-lapse analysis tackles the problem from one of two angles. The first involves the creation of algorithms that identify markers such as pronuclei formation and fading [65, 75, 76], cell divisions [65, 76–79], blastocyst formation and development [65, 76–78, 80], and cytoplasm movement [81]. These markers may then be used downstream by either embryologists or another algorithm to make inferences about the embryo. The second involves the creation of algorithms that directly predict clinical outcomes from timelapses in an end-to-end fashion [34, 43, 44, 51, 53, 54, 82]. This latter group of methods has the advantage that algorithms are free to pick up markers (or interactions between markers) unaccounted

for in standard morphokinetic evaluation. As a result, several such systems have been reported to outperform professional embryologists in the evaluation of time-lapses [34, 82].

AI in gamete and embryo selection: Challenges and opportunities

Data availability

A major challenge experienced by many researchers and practitioners building data-driven AI systems for healthcare is a lack of data [83]. This is no less true for the field of ART. Due to data protection, privacy, or commercial considerations, the data landscape in ART is, at the time of this writing, fragmented and siloed [84]. Moreover, because of the great amount of effort required by embryologists to annotate data sets, the labelled data sets that do exist are often quite small or contain missing labels. This poses a problem for the development of modern data-driven AI systems, which require large, diverse data sets in order to accurately capture variation across the whole patient population. As a result, AI systems that build upon data sets from single clinics may only perform well for a certain clinic's patient demographics. Moreover, the current state of the ART data landscape makes it difficult to verify and reproduce study results.

Nonetheless, in recent years, the field of ART has made its first steps towards a more open data landscape. Several publicly available sperm imaging data sets exist [11, 15, 85]. Gomez et al. [86] released an open data set for benchmarking morphokinetic parameter prediction models. Dirvanauskas et al. [87] tackled the data availability problem from a more technical angle, proposing a generative adversarial network model capable of generating artificial images of embryos that could be used as training data. Progress has also been made towards making effective use of unlabelled data. Kragh et al. [53] used self-supervised learning techniques on unlabelled data to augment the performance of a supervised model. Such an approach allowed the model to be trained on a data set that was only 16% labelled.

The fields of AI and ML have also recently seen several innovations, such as federated learning. Federated learning allows AI models to be trained across multiple devices without transferring each device's data set to a centralized server. As such, federated learning may be used to enable multi-clinic collaborations in which a model is securely trained across data sets held at each clinic, without the need for individual clinics to send each other their own data sets [84]. Another such innovation is differentially private learning, a collection of techniques that allows models to be trained so that they satisfy strong mathematical privacy guarantees [88]. The adoption of such techniques may pave the way towards greater data availability in ART.

Technical challenges

The application of AI to gamete and embryo selection also faces technical hurdles. One such hurdle is the problem of noisy labels. As supervised learning remains the prevailing paradigm for building AI systems for ART, the quality of annotated labels is of great importance. Although these labels are typically provided by expert embryologists, the annotation process is nevertheless noisy. For instance, Khosravi et al. [37] found that a panel of five embryologists grading embryos into three categories ("good," "fair," and "poor") using the Gardner system only agreed on the grades of 89 out of 394 embryos. There are also instances in which noise and uncertainty are inherent in labels obtained from biological processes. Consider, for example, the problem of

attributing a single pregnancy after a multiple embryo transfer to a specific transferred embryo.

Recent studies have taken different approaches to dealing with noisy labels. Khosravi et al. [37] used the majority vote of a panel of embryologists as “gold standard” labels for model training. Erlich et al. [82] addressed the problem of ambiguity in implantation data due to the impact of maternal factors by creating pseudo-labels. These pseudo-labels were generated by using a CNN so that embryos with similar developmental patterns were given the same label. This allowed viable embryos to be identified even if they failed to implant due to maternal factors. Moreover, the method is also applicable to training with unlabelled data.

The ability of AI systems to remain robust across different clinics (known as domain adaptation) presents another hurdle. This is especially problematic for deep learning image analysis models that can be thrown off by variations in imaging set-ups (this can range from different lighting conditions to the use of different imaging apparatus). Common methods used to help improve robustness include data normalization, data augmentation (training models using slightly modified copies of images in the original data set), and data collection from multiple clinics (which has its own challenges, as previously discussed). More recent work has made use of techniques such as adversarial learning, in which an AI system is taught to ignore artefacts specific to a particular imaging set-up. Such an approach enabled Kanakasabapathy et al. [72] to adapt a CNN trained on one image modality (e.g. HMC microscopy) to another (e.g. a smartphone-based imaging system).

Reporting, accountability, and ethical challenges

Various frameworks have been proposed to create unified reporting standards for AI systems in healthcare and other high-impact application domains [89–92]. Among the most popular of these are the guidelines by Collins et al. [92] for the transparent reporting of multivariate prediction models for individual prognosis or diagnosis (TRIPOD). The guidelines are targeted at healthcare publications (to date, no ART-specific reporting guidelines exist) and take the form of a checklist of 22 items to report, including details on the participant population, model development, performance evaluation, and study limitations. However, many AI publications in the ART field do not currently adhere to these TRIPOD guidelines and thus fail to report sufficient information to allow for suitable scrutiny as to the validity of their claims.

Prior to incorporation into clinical practice, it is critical to assess how well AI systems generalize in terms of different patient populations, clinical practices, and rare biological events. For instance, models derived from data sets consisting predominantly of Caucasian populations may see a decrease in performance when used on patients of other ethnicities. Such a model would be a cause for ethical concern because it would lead to inequality in the application of research findings and thus varied success rates with different ethnicities. Moreover, a data-driven AI system may learn to reproduce harmful unconscious biases present in the clinical decisions made while collecting the data set. Thus, there is a need for large, diverse, international testing data sets to allow for AI systems to be suitably validated for generalization and harmful biases.

It is important to highlight that, at this stage, there is no evidence that AI can replace fertility practitioners in clinical decision-making. Instead, the goal of the vast majority of AI systems is to support and simplify clinical decision-making. As such, fertility practitioners continue to be fully accountable for the

advice received from such tools. This presents a particular challenge when the AI system underlying a decision support tool is a so-called “black box”: a system that provides very little visibility or explanation as to how decisions are made. An example of a black box system is a deep neural network that conducts millions of mathematical operations to arrive at a decision. This can lead to concerns about the trustworthiness of AI tools. For instance, when a blastocyst ranking tool proposes that a poor-quality blastocyst be prioritized over a good-quality blastocyst, the embryologist may struggle to follow the AI tool blindly without explanation.

It is therefore imperative that AI tools used in clinical practice provide biologically sound explanations that can be readily understood by clinicians and embryologists. This would help ensure frictionless integration into clinical decision-making processes. For instance, returning to the previous example of a poor-quality blastocyst being prioritized over a good-quality blastocyst, the recommendation of an explainable AI decision support tool might be accompanied by an explanation that despite the first blastocyst’s poor morphology, the second blastocyst had certain morphokinetics outside the normal range and a direct cell division from one to three cells. These parameters would have otherwise been missed by the embryologist had they not been using an AI tool to support embryo selection. Therefore, the understanding, transparency, and explainability of AI decision support tools is essential for their incorporation into clinical practice, especially because the fertility practitioner continues to be accountable for decisions.

Nonetheless, the creation of AI systems that are explainable without compromising predictive power remains an open research problem; very few studies have investigated the use of existing explainability techniques in the context of AI for embryo and gamete selection [39, 47, 56]. Among these is a study by Enatsu et al. [47] who have proposed a CNN-based system in which pregnancy predictions from blastocyst images are accompanied by heatmaps over the image that indicate where the model was “looking.”

Regulatory bodies, particularly in the United States and Europe, are adapting to the introduction of AI into medical devices, and there are a range of guidelines and standards that are currently being introduced into the regulatory pathway. This will help to resolve the risks and challenges associated with AI, and to ensure the safe introduction of this new technology into clinical practice.

The future of AI for gamete and embryo selection

In recent years, the field of ART has made great strides in AI-supported gamete and embryo selection tools. We anticipate that AI will provide a baseline for further technological advances, shifting the way that embryology is practiced away from technical, hands-on processes and towards intellectual processes. This will necessitate a different skill set for the next generation of embryologists. The use of AI may also dramatically enhance the operational efficiencies in the IVF laboratory, increasing embryologists’ capacity for cycles. This will in turn reduce the operational costs of IVF, making it more accessible to the patients who need it.

From a technical standpoint, the landscape of research into AI systems for gamete and embryo selection remains relatively immature when compared to other medical domains, particularly those involving imaging. Recently, the field has seen a trend towards the analysis of videos in lieu of single images,

allowing embryos and gametes to be evaluated over longer time periods and in more detail than currently feasible. Several studies have also begun to analyse or even combine multiple data modalities such as protein profiles and imaging data. It is quite possible that the development of such systems, together with explainability techniques, may prove instrumental in the discovery of new parameters for embryo and gamete selection. Combined with large data sets due to a progressively more open data landscape and technologies such as federated learning, AI may provide insights at a speed and scale that more traditional methods such as randomized control trials (RCTs) have failed to achieve.

Moreover, up until now, studies applying AI to ART have largely been based on supervised learning, which requires large quantities of labelled data. But there are signs of the beginning of a shift away from the paradigm of supervised learning towards the use of semi-supervised, self-supervised, and unsupervised learning techniques. In this way, future studies will likely work with much larger data sets of which only a subset is expertly labelled. The field may also see the use of AI, mathematical modelling, and computer graphics to generate synthetic data and simulations that can reduce the cost of data acquisition and thereby the cost of developing not only AI but also non-AI-based technologies.

The future of AI for gamete and embryo selection is very promising for the field of reproductive medicine.

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References

- Lazzaroni-Tealdi E, Barad DH, Albertini DF, Yu Y, Kushnir VA, Russell H, et al. Oocyte scoring enhances embryo-scoring in predicting pregnancy chances with IVF where it counts most. *PLOS ONE*. 2015;10(12):1–13.
- Basile TMA, Caponetti L, Castellano G, Sforza G. A texture-based image processing approach for the description of human oocyte cytoplasm. *IEEE Trans Instrum Meas*. 2010;59(10):2591–601.
- Aragón J, González AL, Yúfera A. Applying image processing to in-vitro human oocytes characterization. In *Image Processing: Methods, Applications and Challenges*. Carvalho VH (ed.). New York: Nova Publishers, pp 1–17, 2012.
- Manna C, Nanni L, Lumini A, Pappalardo S. Artificial intelligence techniques for embryo and oocyte classification. *Reprod Biomed Online*. 2013;26(1):42–9.
- Baručić D, Kybic J, Teplá O, Topurko Z, Kratochvílová I. Automatic evaluation of human oocyte developmental potential from microscopy images. *arXiv:2103.00302*, 2021.
- Lu Z, Zhang X, Leung C, Esfandiari N, Casper RF, Sun Y. Robotic ICSI (intracytoplasmic sperm injection). *IEEE Trans Biomed Eng*. 2011;58(7):2102–8.
- Firuzinia S, Afzali SM, Ghasemian F, Mirroshandel SA. A robust deep learning-based multiclass segmentation method for analyzing human metaphase II oocyte images. *Comput Methods Programs Biomed*. 2021;201:105946.
- Targosz A, Przystalka P, Wiaderkiewicz R, Mrugacz G. Semantic segmentation of human oocyte images using deep neural networks. *Biomed Eng Online*. 2021;20(1):40.
- Zhang Z, Dai C, Shan G, Chen X, Liu H, Abdalla K, et al. Quantitative selection of single human sperm with high DNA integrity for intracytoplasmic sperm injection. *Fertil Steril*. 2021;116(5):1308–18. <https://doi.org/10.1016/j.fertnstert.2021.06.016>
- Kruger TF, DuToit TC, Franken DR, Acosta AA, Oehninger SC, Menkveld R, et al. A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading. *Fertil Steril*. 1993;59(1):202–9.
- Chang V, Garcia A, Hitschfeld N, Härtel S. Gold-standard for computer-assisted morphological sperm analysis. *Comput Biol Med*. 2017;83:143–50.
- Javadi S, Mirroshandel SA. A novel deep learning method for automatic assessment of human sperm images. *Comput Biol Med*. 2019;109:182–94.
- Kandel ME, Rubessa M, He YR, Schreiber S, Meyers S, Naves LM, et al. Reproductive outcomes predicted by phase imaging with computational specificity of spermatozoon ultrastructure. *Proc Natl Acad Sci U S A*. 2020;117(31):18302–9.
- Riordon J, McCallum C, Sinton D. Deep learning for the classification of human sperm. *Comput Biol Med*. 2019;111:103342.
- Shaker F, Monadjemi SA, Alirezaie J, Naghsh-Nilchi AR. A dictionary learning approach for human sperm heads classification. *Comput Biol Med*. 2017;91:181–90.
- Yüzkat M, İlhan HO, Aydin N. Multi-model CNN fusion for sperm morphology analysis. *Comput Biol Med*. 2021;137:104790.
- İlhan HO, Sigirci IO, Serbes G, Aydin N. A fully automated hybrid human sperm detection and classification system based on mobile-net and the performance comparison with conventional methods. *Med Biol Eng Comput*. 2020;58(5):1047–68.
- Abbasi A, Miah E, Mirroshandel SA. Effect of deep transfer and multi-task learning on sperm abnormality detection. *Comput Biol Med*. 2021;128:104121.
- Chandra S, Gourisaria MK, Gm H, Konar D, Gao X, Wang T, et al. Prolificacy assessment of spermatozoan via state-of-the-art deep learning frameworks. *IEEE Access*. 2022;10:13715–27.
- Movahed RA, Mohammadi E, Ooroji M. Automatic segmentation of sperm's parts in microscopic images of human semen smears using concatenated learning approaches. *Comput Biol Med*. 2019; 109:242–53.
- Marín R, Chang V. Impact of transfer learning for human sperm segmentation using deep learning. *Comput Biol Med*. 2021;136: 104687.
- Valiuškaitė V, Raudonis V, Maskeliūnas R, Damaševičius R, Krilavičius T. Deep learning based evaluation of spermatozoid motility for artificial insemination. *Sensors (Switzerland)*. 2021;21(1):72.
- Alameri M, Hasikin K, Kadri NA, Nasir NFM, Mohandas P, Anni JS, et al. Multistage optimization using a modified Gaussian mixture model in sperm motility tracking. *Comput Math Methods Med*. 2021;2021:6953593.
- Goodson SG, White S, Stevans AM, Bhat S, Kao CY, Jaworski S, et al. CASANova: A multiclass support vector machine model for the classification of human sperm motility patterns. *Biology of Reproduction*. 2017;97(5):698–708.
- Mendizabal-Ruiz G, Chavez-Badiola A, Figueroa IA, Nuño VM, Farias AFS, Valencia-Murillo R, et al. Computer software (SiD) assisted real-time single sperm selection correlates with fertilization and blastocyst formation. *Reprod Biomed Online*. 2022;45(4):703–711. <https://linkinghub.elsevier.com/retrieve/pii/S1472648322002267>
- Sato T, Kishi H, Murakata S, Hayashi Y, Hattori T, Nakazawa S, et al. A new deep-learning model using YOLOv3 to support sperm selection during intracytoplasmic sperm injection procedure. *Reprod Med Biol*. 2022;21(1):e12454. <https://onlinelibrary.wiley.com/doi/10.1002/rmb2.12454>
- Agarwal A, Henkel R, Huang CC, Lee MS. Automation of human semen analysis using a novel artificial intelligence optical microscopic technology. *Andrologia*. 2019;51(11):e13440.
- Hicks SA, Andersen JM, Witczak O, Thambawita V, Halvorsen P, Hammer HL, et al. Machine learning-based analysis of sperm videos and participant data for male fertility prediction. *Scientific Reports*. 2019;9(1):16770.

29. Somasundaram D, Nirmala M. Faster region convolutional neural network and semen tracking algorithm for sperm analysis. *Comput Methods Programs Biomed.* 2021;200:105918.
30. Gunderson SJ, Puga Molina LC, Spies N, Balestrini PA, Buffone MG, Jungheim ES, et al. Machine-learning algorithm incorporating capacitated sperm intracellular pH predicts conventional in vitro fertilization success in normospermic patients. *Fertility and Sterility.* 2021;115(4):930–9.
31. Dimitriadis I, Bormann CL, Kanakasabapathy MK, Thirumalaraju P, Kandula H, Yogesh V, et al. Automated smartphone-based system for measuring sperm viability, DNA fragmentation, and hyaluronic binding assay score. *PLOS ONE.* 2019;14(3):e0212562.
32. McCallum C, Riordon J, Wang Y, Kong T, You JB, Sanner S, et al. Deep learning-based selection of human sperm with high DNA integrity. *Commun Biol.* 2019;2(1):250.
33. Kan-Tor Y, Zabari N, Erlich I, Szekeskin A, Amitai T, Richter D, et al. Automated evaluation of human embryo blastulation and implantation potential using deep-learning. *Adv Intell Syst.* 2020 Oct;2(10):2000080.
34. Liao Q, Zhang Q, Feng X, Huang H, Xu H, Tian B, et al. Development of deep learning algorithms for predicting blastocyst formation and quality by time-lapse monitoring. *Commun Biol.* 2021 Dec 1;4(1):415.
35. Giscard d'Estaing S, Labrune E, Forcellini M, Edel C, Salle B, Lornage J, et al. A machine learning system with reinforcement capacity for predicting the fate of an ART embryo. *Syst Biol Reprod Med.* 2021 Feb;67(1):64–78.
36. Filho ES, Noble JA, Poli M, Griffiths T, Emerson G, Wells D. A method for semi-automatic grading of human blastocyst microscope images. *Hum Reprod.* 2012;27(9):2641–8.
37. Khosravi P, Kazemi E, Zhan Q, Malmsten JE, Toschi M, Zissimopoulos P, et al. Deep learning enables robust assessment and selection of human blastocysts after in vitro fertilization. *NPJ Digit Med.* 2019;2(1):21.
38. Kragh MF, Rimestad J, Berntsen J, Karstoft H. Automatic grading of human blastocysts from time-lapse imaging. *Comput Biol Med.* 2019;115:103494.
39. Thirumalaraju P, Kanakasabapathy MK, Bormann CL, Gupta R, Pooniwala R, Kandula H, et al. Evaluation of deep convolutional neural networks in classifying human embryo images based on their morphological quality. *Heliyon.* 2021 Feb;7(2):e06298.
40. Chavez-Badiola A, Flores-Saiffe-Farias A, Mendizabal-Ruiz G, Drakeley AJ, Cohen J. Embryo ranking intelligent classification algorithm (ERICA): Artificial intelligence clinical assistant predicting embryo ploidy and implantation. *Reprod Biomed Online.* 2020;41(4):585–93.
41. Chen L, Li W, Liu Y, Peng Z, Cai L, Zhang N, et al. Non-invasive embryo selection strategy for clinical IVF to avoid wastage of potentially competent embryos. *Reprod Biomed Online.* 2022 Jul;45(1):26–34.
42. de Gheselle S, Jacques C, Chambost J, Blank C, Declerck K, de Croo I, et al. Machine learning for prediction of euploidy in human embryos: In search of the best-performing model and predictive features. *Fertil Steril.* 2022 Apr 1;117(4):738–46.
43. Huang B, Tan W, Li Z, Jin L. An artificial intelligence model (euploid prediction algorithm) can predict embryo ploidy status based on time-lapse data. *Reprod Biol Endocrinol.* 2021 Dec 1;19(1):185. <https://rbej.biomedcentral.com/articles/10.1186/s12958-021-00864-4>
44. Lee CI, Su YR, Chen CH, Chang TA, Kuo EES, Zheng WL, et al. End-to-end deep learning for recognition of ploidy status using time-lapse videos. *J Assist Reprod Genet.* 2021 Jul 1;38(7):1655–63.
45. Bormann CL, Kanakasabapathy MK, Thirumalaraju P, Gupta R, Pooniwala R, Kandula H, et al. Performance of a deep learning based neural network in the selection of human blastocysts for implantation. *Elife.* 2020;9:1–14.
46. Chavez-Badiola A, Flores-Saiffe Farias A, Mendizabal-Ruiz G, Garcia-Sanchez R, Drakeley AJ, Garcia-Sandoval JP. Predicting pregnancy test results after embryo transfer by image feature extraction and analysis using machine learning. *Sci Rep.* 2020;10(1):4394.
47. Enatsu N, Miyatsuka I, An LM, Inubushi M, Enatsu K, Otsuki J, et al. A novel system based on artificial intelligence for predicting blastocyst viability and visualizing the explanation. *Reprod Med Biol.* 2022;21(1):e12443.
48. Geller J, Collazo I, Pai R, Hendon N, Lokeshwar SD, Arora H, et al. An artificial intelligence-based algorithm for predicting pregnancy success using static images captured by optical light microscopy during intracytoplasmic sperm injection. *J Hum Reprod Sci.* 2021;14(3):288–92.
49. Fitz VW, Kanakasabapathy MK, Thirumalaraju P, Kandula H, Ramirez LB, Boehlein L, et al. Should there be an “AI” in TEAM? Embryologists selection of high implantation potential embryos improves with the aid of an artificial intelligence algorithm. *J Assist Reprod Genet.* 2021 Oct;38(10):2663–70.
50. Alegre L, del Gallego R, Bori L, Loewke K, Maddah M, Aparicio-Ruiz B, et al. Assessment of embryo implantation potential with a cloud-based automatic software. *Reprod Biomed Online.* 2021 Jan;42(1):66–74.
51. Berntsen J, Rimestad J, Lassen JT, Tran D, Kragh MF. Robust and generalizable embryo selection based on artificial intelligence and time-lapse image sequences. *PLoS ONE.* 2022;17(2):e0262661.
52. Huang B, Zheng S, Ma B, Yang Y, Zhang S, Jin L. Using deep learning to predict the outcome of live birth from more than 10,000 embryo data. *BMC Pregnancy Childbirth.* 2022 Jan 16;22(1):36.
53. Kragh MF, Rimestad J, Lassen JT, Berntsen J, Karstoft H. Predicting embryo viability based on self-supervised alignment of time-lapse videos. *IEEE Trans Med Imaging.* 2022;41(2):465–75.
54. Tran D, Cooke S, Illingworth PJ, Gardner DK. Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer. *Hum Reprod.* 2019;34(6):1011–8.
55. ver Milyea M, Hall JMM, Diakiw SM, Johnston A, Nguyen T, Perugini D, et al. Development of an artificial intelligence-based assessment model for prediction of embryo viability using static images captured by optical light microscopy during IVF. *Hum Reprod.* 2020;35(4):770–84.
56. Loewke K, Cho JH, Brumar CD, Maeder-York P, Barash O, Malmsten JE, et al. Characterization of an artificial intelligence model for ranking static images of blastocyst stage embryos. *Fertil Steril.* 2022 Mar;117(3):528–35.
57. Liu R, Bai S, Jiang X, Luo L, Tong X, Zheng S, et al. Multifactor prediction of embryo transfer outcomes based on a machine learning algorithm. *Front Endocrinol (Lausanne).* 2021;12:745039.
58. Amini P, Ramezanali F, Parchehbaf-Kashani M, Maroufizadeh S, Omani-Samani R, Ghaheri A. Factors associated with in vitro fertilization live birth outcome: A comparison of different classification methods. *Int J Fertil Steril.* 2021;15(2):128–34.
59. Bodri D, Milewski R, Yao Serna J, Sugimoto T, Kato R, Matsumoto T, et al. Predicting live birth by combining cleavage and blastocyst-stage time-lapse variables using a hierarchical and a data mining-based statistical model. *Reprod Biol.* 2018 Dec 1;18(4):355–60.
60. Nagaya M, Ukita N. Embryo grading with unreliable labels due to chromosome abnormalities by regularized PU learning with ranking. *IEEE Trans Med Imaging.* 2022;41(2):320–31.
61. Sawada Y, Sato T, Nagaya M, Saito C, Yoshihara H, Banno C, et al. Evaluation of artificial intelligence using time-lapse images of IVF embryos to predict live birth. *Reprod Biomed Online.* 2021 Nov;43(5):843–52.
62. Wu C, Yan W, Li H, Li J, Wang H, Chang S, et al. A classification system of day 3 human embryos using deep learning. *Biomed Signal Process Control.* 2021 Sep 1;70:102943.
63. Xi Q, Yang Q, Wang M, Huang B, Zhang B, Li Z, et al. Individualized embryo selection strategy developed by stacking machine learning model for better in vitro fertilization outcomes: An application study. *Reprod Biol Endocrinol.* 2021 Apr 5;19(1):53.

64. Bori L, Dominguez F, Fernandez EI, Gallego R, Alegre L, Hickman C, et al. An artificial intelligence model based on the proteomic profile of euploid embryos and blastocyst morphology: A preliminary study. *Reprod Biomed Online*. 2020 Feb;42(2):340–50.
65. Leahy BD, Jang WD, Yang HY, Struyven R, Wei D, Sun Z, et al. Automated Measurements of Key Morphological Features of Human Embryos for IVF. In: *Lecture Notes in Computer Science (including subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*. 2020.
66. Rad RM, Saeedi P, Au J, Havelock J. Trophectoderm segmentation in human embryo images via inceptioned U-Net. *Medical Image Analysis*. 2020;62:101612.
67. Rad RM, Saeedi P, Au J, Havelock J. BLAST-NET: Semantic Segmentation of Human Blastocyst Components via Cascaded Atrous Pyramid and Dense Progressive Upsampling. In: *Proceedings - International Conference on Image Processing, ICIP*. 2019.
68. Singh A, Au J, Saeedi P, Havelock J. Automatic segmentation of trophectoderm in microscopic images of human blastocysts. *IEEE Trans Biomed Eng*. 2015;62(1):382–93.
69. Zhao M, Xu M, Li H, Alqawasmeh O, Chung JPW, Li TC, et al. Application of convolutional neural network on early human embryo segmentation during in vitro fertilization. *J Cell Mol Med*. 2021;25(5):2633–44.
70. Saeedi P, Yee D, Au J, Havelock J. Automatic identification of human blastocyst components via texture. *IEEE Trans Biomed Eng*. 2017;64(12):2968–78.
71. Arsalan M, Haider A, Choi J, Park KR. Detecting blastocyst components by artificial intelligence for human embryological analysis to improve success rate of in vitro fertilization. *J Personal Med*. 2022 Feb 1;12(2):124.
72. Kanakasabapathy MK, Thirumalaraju P, Kandula H, Doshi F, Sivakumar AD, Kartik D, et al. Adaptive adversarial neural networks for the analysis of lossy and domain-shifted datasets of medical images. *Nat Biomed Eng*. 2021 Jun 1;5(6):571–85.
73. Raudonis V, Paulauskaite-Taraseviciene A, Sutiene K. Fast multi-focus fusion based on deep learning for early-stage embryo image enhancement. *Sensors (Basel)*. 2021 Jan 28;21(3):863.
74. Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on day 3. *Hum Reprod*. 2016;31(10):2231–44.
75. Fukunaga N, Sanami S, Kitasaka H, Tsuzuki Y, Watanabe H, Kida Y, et al. Development of an automated two pronuclei detection system on time-lapse embryo images using deep learning techniques. *Reprod Med Biol*. 2020;19(3):286–94.
76. Lau T, Ng N, Gingold J, Desai N, McAuley J, Lipton ZC. Embryo Staging with Weakly-Supervised Region Selection and Dynamically-Decoded Predictions. In: Doshi-Velez F, Fackler J, Jung K, Kale D, Ranganath R, Wallace B, et al. (eds.). *Proceedings of the 4th Machine Learning for Healthcare Conference*. PMLR; 2019. pp. 663–79. (*Proceedings of Machine Learning Research*; vol. 106). Available from: <https://proceedings.mlr.press/v106/lau19a.html>
77. Lukyanenko S, Jang WD, Wei D, Struyven R, Kim Y, Leahy B, et al. Developmental stage classification of embryos using two-stream neural network with linear-chain conditional random field. In: *Lecture Notes in Computer Science (Including Subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*. 2021.
78. Lockhart L, Saeedi P, Au J, Havelock J. Automating embryo development stage detection in time-lapse imaging with synergic loss and temporal learning. In: *Lecture Notes in Computer Science (Including Subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*. 2021.
79. Malmsten J, Zaninovic N, Zhan Q, Rosenwaks Z, Shan J. Automated cell division classification in early mouse and human embryos using convolutional neural networks. *Neural Comput Applic*. 2021;33(7):2217–28.
80. Huang TTF, Kosasa T, Walker B, Arnett C, Huang CTF, Yin C, et al. Deep learning neural network analysis of human blastocyst expansion from time-lapse image files. *Reprod Biomed Online*. 2021;42(6):1075–85.
81. Coticchio G, Fiorentino G, Nicora G, Sciajno R, Cavalera F, Bellazzi R, et al. Cytoplasmic movements of the early human embryo: Imaging and artificial intelligence to predict blastocyst development. *Reprod Biomed Online*. 2021 Mar 1;42(3):521–8.
82. Erlich I, Ben-Meir A, Har-Vardi I, Grifo J, Wang F, McCaffrey C, et al. Pseudo contrastive labeling for predicting IVF embryo developmental potential. *Sci Rep*. 2022 Dec 1;12(1):2488.
83. Rajpurkar P, Chen E, Banerjee O, Topol EJ. AI in health and medicine. *Nat Med*. 2022 Jan 20;28(1):31–8.
84. Hickman CFL, Alshubbar H, Chambost J, Jacques C, Pena CA, Drakeley A, et al. Data sharing: Using blockchain and decentralized data technologies to unlock the potential of artificial intelligence: What can assisted reproduction learn from other areas of medicine? *Fertil Steril*. 2020;114:927–33.
85. Haugen TB, Hicks SA, Andersen JM, Witczak O, Hammer HL, Borgli R, et al. VISEM: A multimodal video dataset of human spermatozoa. In: *Proceedings of the 10th ACM Multimedia Systems Conference, MMSys 2019*. Association for Computing Machinery, Inc. 2019. pp. 261–6.
86. Gomez T, Feyeux M, Normand N, David L, Paul-Gilloteaux P, Fréour T, et al. Towards deep learning-powered IVF: A large public benchmark for morphokinetic parameter prediction. 2022 Mar 1. <http://arxiv.org/abs/2203.00531>
87. Dirvanauskas D, Maskeliūnas R, Raudonis V, Damaševičius R, Scherer R. HEMIGEN: Human embryo image generator based on generative adversarial networks. *Sensors (Switzerland)*. 2019;19(16):3578.
88. Ficek J, Wang W, Chen H, Dagne G, Daley E. Differential privacy in health research: A scoping review. *J Am Med Inform Assoc*. 2021;28(10):2269–76.
89. Wolff RF, Moons KGM, Riley RD, Whiting PF, Westwood M, Collins GS, et al. PROBAST: A tool to assess the risk of bias and applicability of prediction model studies. *Ann Intern Med*. 2019;170(1):51–8.
90. Mitchell M, Wu S, Zaldivar A, Barnes P, Vasserman L, Hutchinson B, et al. Model cards for model reporting. In: *FAT* 2019 - Proceedings of the 2019 Conference on Fairness, Accountability, and Transparency*. 2019.
91. Hernandez-Boussard T, Bozkurt S, Ioannidis JPA, Shah NH. MINIMAR (MINIMUM information for medical AI reporting): Developing reporting standards for artificial intelligence in health care. *J Am Med Inform Assoc*. 2020; 27:2011–15.
92. Collins GS, Reitsma JB, Altman DG, Moons KGM. Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD): The TRIPOD statement. *European Urology*. 2015;67(6):W1–73.

20

DEMYSTIFYING VITRIFICATION

Debra A. Gook and Kelly Lewis

Introduction

The theory and results from the application of vitrification in assisted reproductive technology (ART) have been extensively covered elsewhere [1–8]. The aim of this chapter is not to repeat this body of work, but to discuss the technical issues that arise while vitrifying biological material in ART. It will also put these issues into context by providing evidence relating to their role as critical parameters and provide practical information to assist in achieving optimal vitrification outcomes.

By combining more than 30 years' experience in cryopreservation (including teaching and watching many scientists performing vitrification in the laboratory) with theoretical knowledge, this chapter will provide the basis for understanding why some small variations in methodology can have an impact on the outcomes of vitrification. The information provided is applicable to the vitrification of both human oocytes and embryos, although much of the experimental data has been generated using discarded human oocytes (metaphase I [MI] and germinal vesicle [GV] stage oocytes matured overnight to the metaphase II [MII] stage) and aims to assist in eliminating user variation and achieving reproducibly high outcomes with vitrification.

Cryopreservation, regardless of whether using a controlled rate of cooling (often referred to a slow cooling) or vitrification, requires the expulsion of intracellular water prior to cooling and replacing it with a permeable cryoprotectant. The aim of both approaches is to have no free water and a minimal amount of bound water which could form ice during cooling or warming. Most commercial vitrification kits on the market are composed of three solutions: (i) a buffer solution without cryoprotectant; (ii) a solution containing approximately a 3 M concentration of two permeating cryoprotectants, often dimethyl sulfoxide (DMSO) and ethylene glycol (EG), which will be referred throughout as the equilibration solution (ES); and (iii) a solution containing double the concentration of permeating cryoprotectants in the ES with the addition of a non-permeating molecule, generally a sugar such as sucrose or trehalose, which will be referred to as the vitrification solution (VS) throughout. To reduce individual cryoprotectant toxicity at high concentrations, a combination of two cryoprotectants has the benefit of achieving the required dehydration while reducing toxicity [9]. Regardless of the developmental stage, i.e. oocytes, cleavage stage embryos, or blastocysts, most kits are recommended for universal application and only vary in the recommended time spent in the solutions in each case. This relates to the size of the cell and therefore the water content within that cell.

Oocyte vitrification

The impetus for many clinics to attempt to vitrify human oocytes came from the high survival rates achieved with donor oocytes reported by Cobo 2010 using the Kitazato kit [10]. The method

originally reported by Cobo 2008 [11] is described in Figures 20.1 and 20.2. This will be referred to as the standard procedure when comparing parameters throughout this chapter. However, when this approach was applied to oocytes from infertile women, survival rates were more variable [12–14]. As with much of ART, the likely explanation was thought to be female age, but comparison of survival rates relative to female age showed no significant effect other than a trend towards slightly lower survival with advanced age [15]. Survival rates for oocytes from young (<38-year-old) and older (>38-year-old) infertile women were also not significantly different in our vitrification system [16]. In contrast, others have shown age to be a significant factor [17]. Oocyte quality is also more variable in infertile women, potentially impacting on survival, but this was also reported to not be responsible for reduced survival [18].

Dehydration

What is variable across a cohort of oocytes is the rate at which water moves, referred to as the hydraulic permeability coefficient [19], which is temperature-dependent. This can vary within a cohort by as much as eightfold between individual oocytes from the same stimulation cycle [20], implying that there will be variability in the level of dehydration achieved in the ES when applied for a set time. The time at which an oocyte re-expands in the ES solution to 80% of its initial volume is an indication of the hydraulic permeability coefficient for that oocyte. We have measured this for a large number of oocytes (Table 20.1) and confirmed this observation of variability between individual oocytes from a single cohort/patient cycle. However, no relationship was observed between the re-expansion time and maturation stage or re-expansion time and patient age. In the standard method (Figure 20.1) oocytes were moved out of the ES when re-expansion was achieved, thereby allowing for this variation, but commercial methods recommend a set time in the ES solution. In our experiments, although survival rates for those transferred to VS once re-expanded in ES compared to those following a set time of 10 minutes in ES were not significantly different (re-expansion 92.4% [73/79]; set 10 minutes 84.0% [79/94]), there is a suggestion that fewer oocytes have survived with 10 minutes in ES, and that a proportion may require slightly longer to remove all of the water.

In the case of blastocysts, although inner cell mass (ICM) cells are more similar in size and therefore water content, there will be a concentration gradient of water and cryoprotectant across the ICM. This implies that the position of individual ICM cells relative to the zona pellucida and cavity will dictate the time required for each cell to dehydrate, indicating again, that a set time in ES may not be appropriate for all cells within blastocysts and that any recommended time in ES at a particular temperature is likely to be a compromise. With respect to the blastocyst, this will be explored further in the “to collapse or not” section later in the chapter.

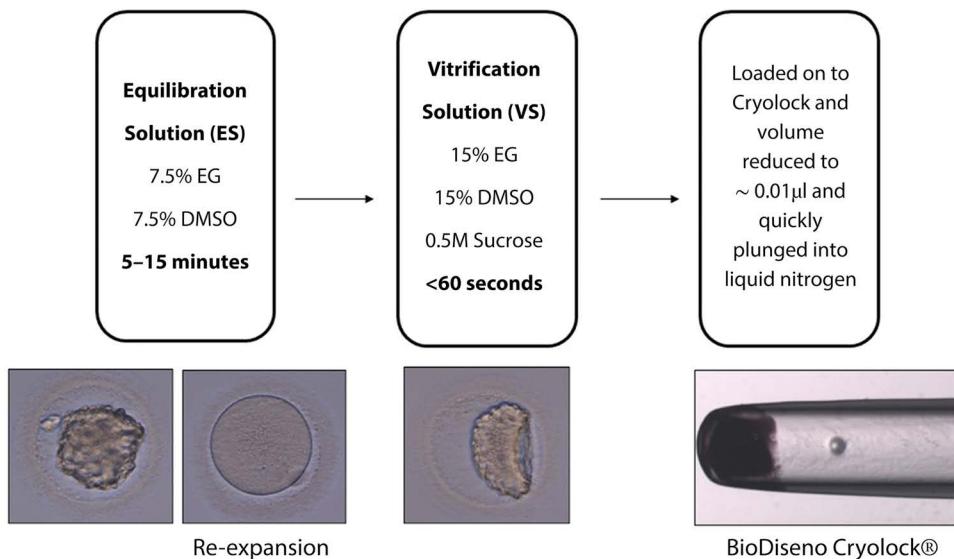


FIGURE 20.1 Oocyte vitrification method. All procedures performed at room temperature. (Kuwayama M, Vajta G, Kato O, Leibo SP, Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online. 2005 Sep;11(3):300–8. doi: 10.1016/s1472-6483(10)60837-1; modified by [11]).

It is also worth noting that increased variation in methodology for the ES exposure, such as the drop merging system or floating on the surface of the ES, will increase variability in the extent of dehydration.

In the VS the concentration gradient results in further dehydration. How quickly this is achieved is dependent on the amount of intracellular water remaining after the ES exposure. The technical ability of the embryologist is also an important factor and in some cases concern regarding the high concentration of cryoprotectant in VS has prompted a tendency to only expose oocytes to VS for as short a time as possible. Again, for a large cell such as the oocyte this is counterintuitive, since the oocyte will still contain water after exposure to the ES and will, therefore, require additional time in the VS solution. Analysis of the impact of duration in the VS has shown that a longer time of 90 seconds in VS results

in similar survival to the standard method (Figure 20.1) (100% [20/20]). In contrast, a longer time of 80 seconds in the VS followed by holding the cryolock in air for 10 seconds before plunging has a significantly negative impact on survival (71% [15/21]). This scenario is designed to mimic the situation when too much solution is pipetted onto the cryolock and there is a delay before plunging while removing the excess VS surrounding the oocyte. Knowledge that this has an impact prompts the next question, as to whether the smallest volume is required on the cryolock to achieve the cooling rate required to successfully vitrify oocytes.

Cooling rate

The volume of VS surrounding the oocyte or blastocyst on the cryolock has been reported to be the most critical parameter

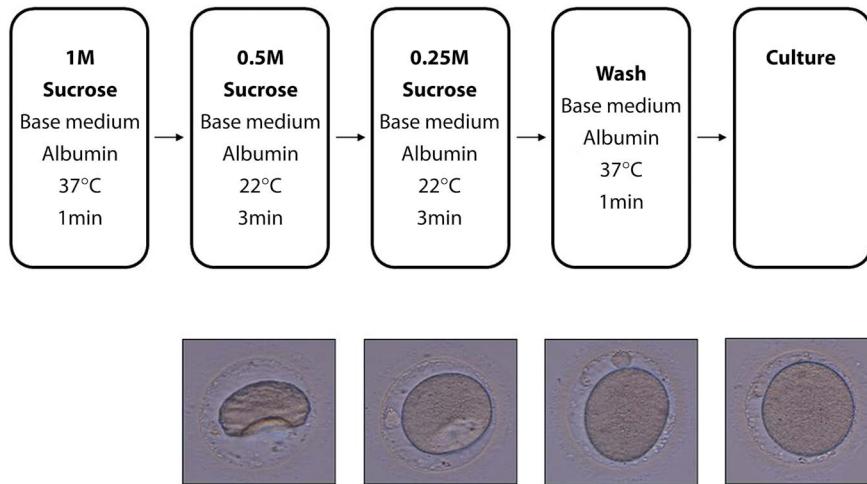


FIGURE 20.2 Oocyte warming.

TABLE 20.1 Re-expansion Time for Oocytes

Oocyte	Mean (Minutes)	Range (Minutes)
GV → MII	6.45	5.0–10.15
MI → MII	7.1	5.2–9.25
MII (<37 year) (n = 2293)	6.7	4.0–12.0
MII (≥37 year) (n = 1317)	6.5	3.5–11.0

in achieving the ultra-high cooling rate required for successful vitrification [21]. The risk of excess solution increases when vitrifying multiple oocytes on the one tool; therefore, to minimize this the excess solution is usually aspirated from around the oocyte before plunging. To assess the effect of a lower cooling rate, an excess of 0.5 µL of VS around the oocyte was tested and no impact on survival (95.2% [20/21]) was observed. This indicates that a slower cooling rate, at least as associated with this amount of excess volume, was not the critical parameter it was previously thought to be, and that exposure to air when removing excess VS had a greater impact on survival.

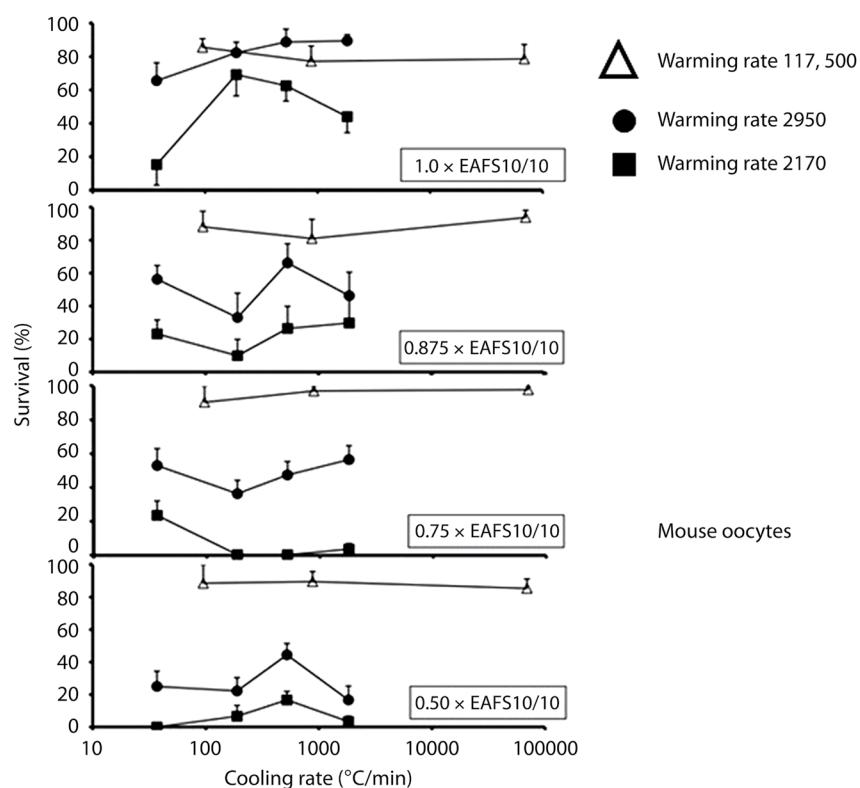
In fact, a slower cooling rate of $-1220^{\circ}\text{C}/\text{minute}$ achieved with a closed vitrification system (Rapid-I), which vitrifies using super-cooled air, can also achieve high survival rates with oocytes [16] that are not significantly different to those achieved with the standard open system (Figure 20.1). The oocyte is loaded in a hole on the Rapid-I that holds 30 nl of VS. This tiny volume of fluid facilitates in achieving the cooling rate reported with super-cooled air. Outside the hole, the plastic is reasonably thick and placing an

oocyte on this area in a relatively large volume of 0.5 µL of VS had an impact on survival (85.7% [30/35]), suggesting that a slower cooling rate than $-1220^{\circ}\text{C}/\text{minute}$ is less favourable for oocyte vitrification.

Warming rate

Seki and Mazur [22] clearly showed with mouse oocytes that an extremely rapid warming rate—not the cooling rate—is the critical parameter. They showed, regardless of the cryoprotectant concentration and slower cooling rates (100 and $1000^{\circ}\text{C}/\text{min}$), that high survival can be achieved when the speed of warming is $117,500^{\circ}\text{C}/\text{minute}$ (Figure 20.3). To achieve the extremely rapid warming rate, the tool is plunged directly into the first warm solution at 37°C and this is fundamental to achieving high survival rates. Therefore, the first warming solution should be warmed to 37°C for sufficient time to establish even temperature throughout the solution and verified in-house with dishes used for warming.

There is a risk of transient warming at any time after vitrification when tools have not been maintained under liquid nitrogen. Due to the low thermal mass of the vitrified tools, and the process required to load and unload transport tanks, there is a high-risk of temperature fluctuations during the transport process, which has been shown to impact on survival [23]. Comparison of exposure to a mock process involved with transporting and receiving vitrified oocytes (Figure 20.3), to vitrified oocytes not exposed to the transport system (i.e. remaining in storage tank until warming), showed similar initial survival rates, but with time in culture (24 hours) the mock transport oocytes' survival deteriorated and an increase in spindle abnormalities was observed within these oocytes. In contrast, survival was similar for those kept in storage

**FIGURE 20.3** The rate of warming may be more critical to successful vitrification than cooling.

and the mock transport when liquid nitrogen was decanted into the transport tank before removing the goblets.

To assess the impact of temperature, goblets containing vitrified oocytes were exposed to different temperatures in a transport tank. The temperature within a transport tank will start to increase over time (Figure 20.4), providing a sub-zero temperature range that the goblet contents can be exposed to but remain cryopreserved, after which the goblet is quickly returned to liquid nitrogen for warming at a later time. Using this system, the survival of oocytes exposed to a range of sub-zero temperatures can be compared (Figure 20.5). A significant ($p < 0.001$) reduction

in survival was observed with a brief exposure to -63.8°C and -53.8°C suggesting that, at these temperatures, there has been a transient devitrification and, on returning to storage under liquid nitrogen, a revitrification. Whether this damage is a consequence of devitrification or revitrification is unknown.

Rehydration

There are two general methods employed to rehydrate vitrified ART cells, i.e. by (i) exposure to a high concentration of non-permeating cryoprotectant only and (ii) exposure to reduced

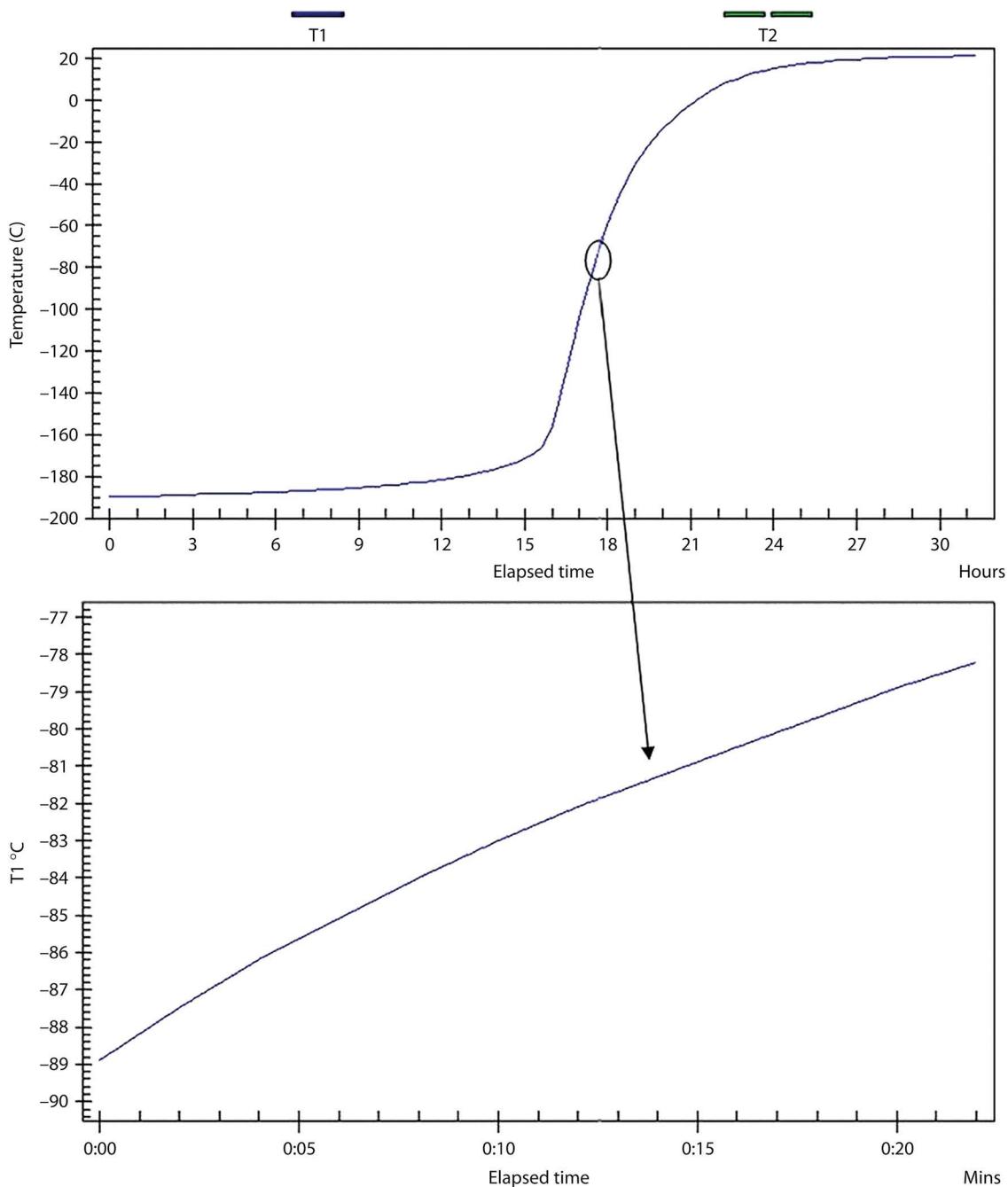


FIGURE 20.4 Temperature during dry shipper during warmer phase. Temperature range -88.9°C to -78.2°C ; time 22 minutes; mean temperature -83.1°C .

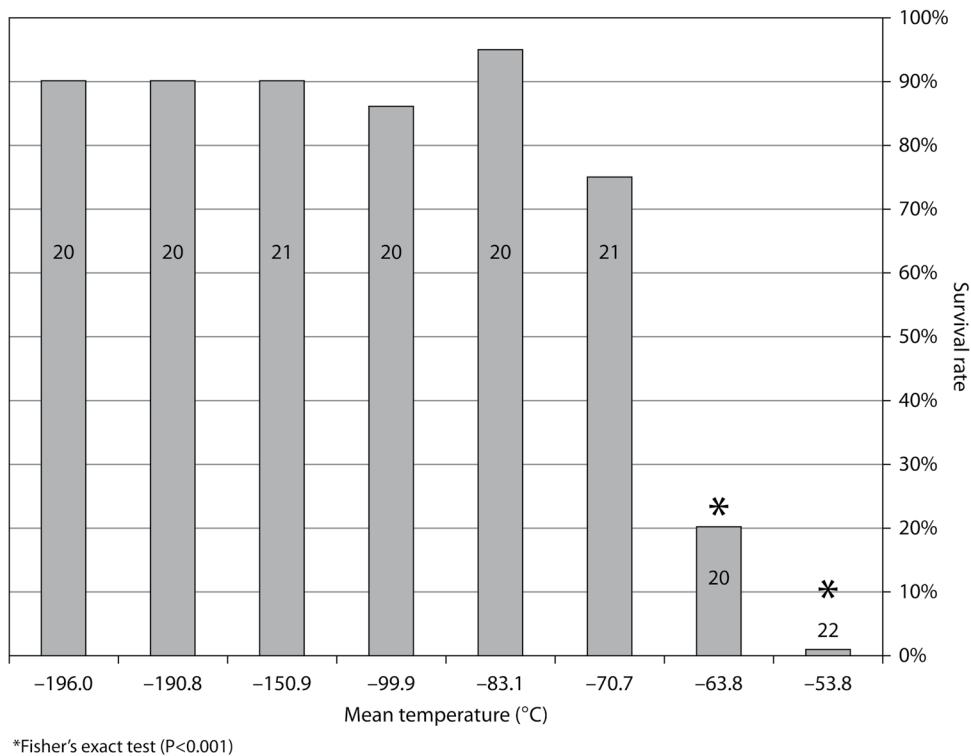


FIGURE 20.5 Oocyte survival rates after temperature exposure.

concentrations of permeating cryoprotectants with a higher concentration of non-permeating cryoprotectant, both followed by transfer to basal medium. The first rehydration method, used by Cobo 2008 (Figure 20.2), is the principle used in most commercial warming kits and prescribes 60 seconds in the high non-permeating (generally 1M sucrose) solution. How critical the time is in this solution was examined and a reduction in survival was observed when oocytes were exposed for only 30 seconds (64.0%) and also for a longer time (90 seconds; 48.8%). Both were significantly lower than when exposure was for 60 seconds ($p < 0.01$ for both compared to 60 seconds; 92.4%).

In the Cobo 2008 procedure, and most commercial kits, the subsequent solution is half the concentration of non-permeating cryoprotectant (generally sucrose) but for this solution the exposure is now at room temperature. It is difficult to understand the rationale for this change in temperature, apart from the fact that

the hydraulic permeability coefficient is temperature-dependent and, therefore, the rate of permeating cryoprotectant moving out of the oocyte at room temperature is slower. However, at this high non-permeating concentration (0.5 M) there is probably very little water and permeating cryoprotectant movement. Changing the temperature of this solution to 37°C had no impact on survival when exposure time was reduced to 1 minute in our experimental procedure (100% [12/12]) and with clinical vitrified oocytes donated to research (100% [30/30]). This approach has the added benefit that all solutions in the warming process are at 37°C physiological temperature. This has been confirmed for oocytes showing high survival, fertilization, and implantation rates when all warming steps are at 37°C [24].

A frequently asked question is whether a universal warming protocol is appropriate to warm oocytes and blastocysts vitrified with a variety of kits (Table 20.2). A crossover study of

TABLE 20.2 Broad Composition of Vitrification Kits

Vitrification Kit	EG	DMSO	PROH	Sucrose	Trehalose	Other
Kitazato ^a	X	X			X	Hydroxypropyl cellulose
SAGE	X	X		X		
Irvine	X	X		X		
COOK	X	X			X	
Medicult	X		X	X		
VITROLIFE Cleave and OMNI	X		X	X		Ficoll Hyaluronan

Note:

^a Initial composition of the Kitazato kit was sucrose and synthetic serum substitute; since 2016 these have been replaced with trehalose and hydroxypropyl cellulose.

Kitazato and SAGE blastocyst vitrification kits and in-house warming solutions of 1.0 M sucrose, 0.5 M sucrose, showed no difference in blastocyst survival and implantation rates, which were similar to fresh [25]. Both vitrification kits consist of the same permeating cryoprotectants, and their concentrations are the same, but vary in the base buffer and the non-permeating cryoprotectant (see Table 20.2). Considering that the dilution from medium on the vitrification tool is minuscule, and that the non-permeating cryoprotectants are only outside the blastocyst, it is not surprising that the outcomes are the same. This has been repeated in another clinical study [26] with similar composition kits and no difference was observed. However, there are major differences in composition with other kits; the COOK kit has a different non-permeating cryoprotectant (trehalose 0.68 M) and slightly higher concentrations of permeating cryoprotectants (both EG and DMSO at 16%), and the Vitrolife vitrification kits (blastocyst, cleavage, and OMNI) have propanediol and EG (both 16%) as the permeating cryoprotectants and 0.65 M sucrose, Ficoll, and hyaluronan, which all act as non-permeating cryoprotectants (Table 20.2). A comparison of mouse blastocysts vitrified with Sage (same composition as Figure 20.1), COOK, or Vitrolife kits and warmed with the same kit warming solution or the universal warming kit (sucrose) showed no difference in survival in the paired comparison for both Cook and Vitrolife initially and 10 hours post warming (unpublished data Moazzam 2021). However, in contrast to the Parmegiani 2018 study, SAGE vitrification with SAGE warming was significantly better than with the universal warming ($p < 0.05$). This is surprising considering both have 1.0 M sucrose followed by 0.5 M sucrose as their warm solutions with the same timing and temperature (Table 20.3). Time to full re-expansion was similar across all groups (three to four hours) with the exception of those vitrified with the Vitrolife kit and warmed with the universal warm solutions, which took significantly ($p < 0.05$) longer to re-expand (7.5 hours). A similar study (unpublished data Aarshiya 2018) with mouse oocytes vitrified with SAGE, Vitrolife (OMNI) kits and an in-house trehalose vitrification kit, all with matched warming solutions or the universal solution showed no difference. There was a suggestion of reduced survival with the Vitrolife vitrification solutions regardless of warming solutions but numbers were insufficient to show a statistical difference. Subsequent fertilization and blastocyst development were similar across all groups. This concept requires further assessment but may indicate the potential of a universal warming method.

To collapse or not

In the early days of vitrification, there were a number of publications describing various methods for collapsing the blastocoel cavity prior to dehydration [27–33] and reports on the value of artificial shrinkage have continued to appear in the literature [34–37]. Regardless of how the cavity size was reduced—needle, laser, or micropipette [29]—post-vitrification survival was improved following the artificial collapsing of the cavity compared to no intervention [27, 28]. In many groups, breeching the zona and trophectoderm continues to be performed and is reported to be superior to vitrifying expanded blastocysts [38]. The process of reducing the fluid cavity artificially will reduce the water and permit faster dehydration of the cells on the inner side of the ICM. However, the cavity also contains other components [39] that will also leak out due to the rupture and these will not be replaced during warming. This approach may be adopted to increase the rate of dehydration or to promote subsequent implantation (i.e. assisted hatching), in the latter case based on an unsubstantiated belief that zona hardening occurs as a result of vitrification [40]. Irrespective of the aim, some of the contents of the cavity will be lost. As stated earlier in the dehydration section, the aim is to remove all free water prior to vitrification and, therefore, it is vital to reduce the size of the cavity so that cells will not be ruptured by ice during vitrification and/or warming. Many cavities will collapse by themselves in the ES solution without assistance, but those cannot be predicted on the basis of blastocyst morphology. Therefore, collapsing the cavity by the aforementioned methods or pipetting [41, 42] during the time in ES will facilitate movement of water and dehydration prior to moving to the VS solution in which dehydration is completed. Similarly, vitrifying biopsied blastocysts shortly after biopsy while collapsing has been shown to be beneficial [38].

Conclusion

As we increase our knowledge of the critical factors affecting vitrification, our ability to achieve high survival levels should also increase. It then becomes a matter of the quality of training of staff in what is required to achieve high survival. This should be complemented by promoting and developing an understanding of the principles underlying the kit composition, the method, and the critical parameters. Regular refresher courses will also be important to maintain KPIs and ensure that individual drift in methodology has not occurred.

TABLE 20.3 Broad Composition of Warming Kits

Warming Kit	EG	DMSO	PROH	Sucrose ^a	Trehalose	Other
Kitazato ^b					X	Hydroxypropyl cellulose
SAGE				X		
Medicult				X		
COOK					X	
VITROLIFE Blast, Cleave and OMNI				X		Hyaluronan

Notes:

^a Sucrose concentration is 1.0 M except for VITROLIFE Blastocyst and Cleave Kits (<1.0 M).

^b Initial composition of the Kitazato kit was sucrose and synthetic serum substitute; since 2016 these have been replaced with trehalose and hydroxypropyl cellulose.

References

1. Leibo SP. Cryopreservation of oocytes and embryos: Optimization by theoretical versus empirical analysis. *Theriogenology*. 2008; 69(1):37–47.
2. Mazur P. Cryobiology: The freezing of biological systems. *Science*. 1970;168(934):939–49.
3. Mazur P, Rall WF, Leibo SP. Kinetics of water loss and the likelihood of intracellular freezing in mouse ova. Influence of the method of calculating the temperature dependence of water permeability. *Cell Biophys*. 1984;6(3):197–213.
4. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. *Nature*. 1985;313(6003):573–5.
5. Vajta G, Kuwayama M. Improving cryopreservation systems. *Theriogenology*. 2006;65(1):236–44.
6. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online*. 2006;12(6):779–96.
7. Rienzi L, Gracia C, Maggiulli R, et al. Oocyte, embryo and blastocyst cryopreservation in ART: Systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update*. 2017;23(2):139–55.
8. Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Hum Reprod Update*. 2012;18:536–54.
9. Ali J, Shelton JN. Design of vitrification solutions for the cryopreservation of embryos. *J Reprod Fertil*. 1993;99(2):471–7.
10. Cobo A, Meseguer M, Remohi J, et al. Use of cryo-banked oocytes in an ovum donation programme: A prospective, randomized, controlled, clinical trial. *Hum Reprod*. 2010;25(9):2239–46.
11. Cobo A, Kuwayama M, Perez S, et al. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertil Steril*. 2008;89(6):1657–64.
12. Herrero L, Pareja S, Aragones M, et al. Oocyte versus embryo vitrification for delayed embryo transfer: An observational study. *Reprod Biomed Online*. 2014;29(5):567–72.
13. Rienzi L, Romano S, Albricci L, et al. Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: A prospective randomized sibling-oocyte study. *Hum Reprod*. 2010;25(1):66–73.
14. Ubaldi F, Anniballo R, Romano S, et al. Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program. *Hum Reprod*. 2010;25(5):1199–205.
15. Cobo A, Garcia-Velasco JA, Coello A, et al. Oocyte vitrification as an efficient option for elective fertility preservation. *Fertil Steril*. 2016;105(3):755–64 e8.
16. Gook DA, Choo B, Bourne H, et al. Closed vitrification of human oocytes and blastocysts: Outcomes from a series of clinical cases. *J Assist Reprod Genet*. 2016;33(9):1247–52.
17. Nagy ZP, Anderson RE, Feinberg EC, et al. The human oocyte preservation experience (HOPE) registry: Evaluation of cryopreservation techniques and oocyte source on outcomes. *Reprod Biol Endocrinol*. 2017;15(1):10.
18. Coello A, Sanchez E, Vallejo B, et al. Effect of oocyte morphology on post-warming survival and embryo development in vitrified autologous oocytes. *Reprod Biomed Online*. 2019;38(3):313–20.
19. Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J Gen Physiol*. 1963;47:347–69.
20. Hunter JE, Bernard A, Fuller BJ, et al. Measurements of the membrane water permeability (L_p) and its temperature dependence (activation energy) in human fresh and failed-to-fertilize oocytes and mouse oocyte. *Cryobiology*. 1992;29(2):240–9.
21. Vajta G, Rienzi L, Ubaldi FM. Open versus closed systems for vitrification of human oocytes and embryos. *Reprod Biomed Online*. 2015;30(4):325–33.
22. Seki S, Mazur P. Ultra-rapid warming yields high survival of mouse oocytes cooled to -196 degrees c in dilutions of a standard vitrification solution. *PLoS One*. 2012;7(4):e36058.
23. McDonald CA, Valluzo L, Chuang L, et al. Nitrogen vapor shipment of vitrified oocytes: Time for caution. *Fertil Steril*. 2011;95:2628–30.
24. Labrado C, Navarro JM. Clinical validation supports the concept of universal warming protocols for vitrified human oocytes. *Fertil Steril*. 2020;114(3):e161.
25. Parmegiani L, Beilby KH, Arnone A, et al. Testing the efficacy and efficiency of a single "universal warming protocol" for vitrified human embryos: Prospective randomized controlled trial and retrospective longitudinal cohort study. *J Assist Reprod Genet*. 2018;35(10):1887–95.
26. Parmegiani L, Minasi MG, Arnone A, et al. "Universal warming" protocol for vitrified oocytes to streamline cell exchange for trans-national donation programs: A multi-center study. *J Assist Reprod Genet*. 2020;37(6):1379–85.
27. Mukaida T, Oka C, Goto T, Takahashi K. Artificial shrinkage of blastocoels using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. *Hum Reprod*. 2006;21(12):3246–52.
28. Vanderzwalmen P, Bertin G, Debauche C, et al. Births after vitrification at morula and blastocyst stages: Effect of artificial reduction of the blastocoel cavity before vitrification. *Hum Reprod*. 2002;17(3):744–51.
29. Iwayama H, Hochi S, Yamashita M. In vitro and in vivo viability of human blastocysts collapsed by laser pulse or osmotic shock prior to vitrification. *J Assist Reprod Genet*. 2011;28(4):355–61.
30. Lee SY, Kim HJ, Park SJ. Optimization of a dilution method for human expanded blastocysts vitrified using EM grids after artificial shrinkage. *J Assist Reprod Gen*. 2006;23:87–91.
31. Son WY, Yoon SH, Yoon HJ, et al. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. *Hum Reprod*. 2003;18(1):137–9.
32. Chen SU, Lee TH, Lien YR, et al. Microsuction of blastocoelic fluid before vitrification increased survival and pregnancy of mouse expanded blastocysts, but pretreatment with the cytoskeletal stabilizer did not increase blastocyst survival. *Fertil Steril*. 2005;84(Suppl 2):1156–62.
33. Desai NN, Szeptycki J, Scott M, et al. Artificial collapse of blastocysts before vitrification: Mechanical vs. laser technique and effect on survival, cell number, and cell death in early and expanded blastocysts. *Cell Preserv Technol*. 2008;6:181–90.
34. Van Landuyt L, Polyzos NP, De Munck N, et al. A prospective randomized controlled trial investigating the effect of artificial shrinkage (collapse) on the implantation potential of vitrified blastocysts. *Hum Reprod*. 2015;30(11):2509–18.
35. Ebner T, Shebl O. Artificial blastocoel collapse of human blastocysts before vitrification and its effect on re-expansion after warming. *Reprod Biomed Online*. 2018;36(6):627.
36. Kovacic B, Taborin M, Vlaisavljevic V. Artificial blastocoel collapse of human blastocysts before vitrification and its effect on re-expansion after warming – a prospective observational study using time-lapse microscopy. *Reprod Biomed Online*. 2018;36(2):121–9.
37. Mitsuhasha S, Endo Y, Hayashi M, et al. Effect on clinical and neonatal outcomes of blastocellic microsuction prior to vitrification. *Reprod Med Biol*. 2019;18(3):284–9.
38. Cimadomo D, Capalbo A, Levi-Setti PE, et al. Associations of blastocyst features, trophectoderm biopsy and other laboratory practice with post-warming behavior and implantation. *Hum Reprod*. 2018;33(11):1992–2001.
39. Palini S, Galluzzi L, De Stefani S, et al. Genomic DNA in human blastocoel fluid. *Reprod Biomed Online*. 2013;26(6):603–10.

40. Endo Y, Mitsuhasha S, Hayashi M, et al. Laser-assisted hatching on clinical and neonatal outcomes in patients undergoing single vitrified blastocyst transfer: A propensity score-matched study. *Reprod Med Biol.* 2021;20(2):182–9.
41. Hiraoka K, Kinutani M. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Hum Reprod.* 2004;19(12):2884–8.
42. Coello A, Meseguer M, Galan A, et al. Analysis of the morphological dynamics of blastocysts after vitrification/warming: Defining new predictive variables of implantation. *Fertil Steril.* 2017;108(4):659–66 e4.

VITRIFICATION OF THE HUMAN OOCYTE

Masashige Kuwayama

Introduction

The past 50 years have yielded impressive breakthroughs in cryopreservation as applied to the discipline of reproductive biology. Techniques were usually derived in experimental and domestic animals, and subsequently applied to humans. The first success in freezing cells was achieved in spermatozoa [1], followed by successful cryopreservation of pre-implantation embryos at different stages of development [2–4]. Since the first report in 1972 of cryopreservation of mammalian embryos resulting in the birth of live mice offspring [2], attempts to cryopreserve human oocytes, similar to the results with oocytes of domestic animals, mostly failed for many years. However, the development of an ultra-rapid vitrification method now means that oocytes can be cryopreserved without significant loss of their viability, and such oocytes may be used clinically [5].

The reasons to cryopreserve human oocytes are widely known and were summarized recently [6, 7]. Common indications for this procedure include diseases and their treatments, i.e. to preserve the reproductive competence of unmarried young women with cancer who need irradiation of the pelvic region or chemotherapy, or who require surgical intervention before or during their reproductive age that may involve removal of ovaries. Another reason for cryopreservation is when patients have problems resulting from ovarian malfunction, including premature menopause, ovarian hyperstimulation syndrome, or poor response to ovarian stimulation. There are also legal, ethical, social, and practical problems that may also require oocyte cryopreservation: some countries restrict or prohibit embryo cryopreservation which only leaves the option to preserve oocytes; women may wish to delay motherhood for various reasons, such as career priorities; and there may cases where there is no semen available after a successful oocyte retrieval, to mention a few examples.

However, as discussed in detail recently [6–9], in broader terms, oocyte cryopreservation is also needed to compensate for the unique situation of women in regards to reproduction. As in most mammalian species, women suffer more and sacrifice more for their offspring both physically and emotionally. Yet, a woman's reproductive capability is restricted in terms of quantity and duration. Males produce hundreds of millions of sperm in a single ejaculation, while females ovulate normally only one oocytes every 28 days. From the time that he reaches puberty, a man's reproductive capability is almost unlimited, while that of a woman (without considering special treatments) is limited to a period of just 15 to 20 years. Assisted reproductive techniques did not eliminate this difference. In fact, with the introduction of the procedure of intracytoplasmic sperm injection (ICSI) and successful cryopreservation of sperm, the gap has widened considerably. Apart from the practical goals, our moral duty is to help develop an efficient and safe oocyte cryopreservation method to enhance the reproductive capability of women.

Unfortunately, the task is rather demanding. Although the first pregnancy from a cryopreserved oocyte was reported

about 40 years ago [10], advances until recently were very slow. Generally, inefficiency and lack of consistency were the two main problems [11]. Oocytes are unique cells; their large size, spherical shape, low membrane permeability, and general fragility explain many of the difficulties that occur during cryopreservation.

Oocytes are often described as the largest cells of the mammalian body, and this represents a real challenge in cryopreservation. Cell volume is known to be a crucial parameter that determines the likelihood of success when a cell is cryopreserved. Viruses and bacteria, which have a very tiny volume, may survive deep freezing without any special treatment, such as use of cryo-protectants or controlled rate cooling. Freezing of fibroblasts or epidermal cells is usually an easy and efficient routine task in tissue culture laboratories, and does not need any special instruments. Sperm cryopreservation can be efficiently performed with the use of a controlled-rate freezer. Early cleavage-stage embryos with individual blastomeres having 50% to as little as 10% of the original size of oocytes survive traditional slow-rate freezing very well, and their developmental competence is usually well preserved. Preantral and primary follicles can also be frozen successfully, in contrast to the large, fully developed, metaphase II-stage (MII) oocytes.

The near-spherical shape of the oocyte does not confer an advantage from the point of view of cryopreservation. During equilibration and dilution before and after cooling and warming, permeable cryo-protectants must be distributed rapidly and uniformly throughout the ooplasm. A large spherical object, such as an oocyte, has the lowest surface area/volume ratio of any geometric shape. An irregular object, such as a fibroblast or lymphocyte, has a much larger surface area/volume ratio and will equilibrate osmotically much faster than an oocyte.

The one cell-stage of an oocyte also severely limits options, as there is no margin for error. The single cell survives or it does not. Multicellular embryos may survive and develop even if more than 50% of their cells are damaged. This fact is clearly demonstrated by successful births resulting from bisected embryos of domestic animals.

However, apart from the size, shape, and cell number, other factors may also play an important role in limiting successful oocyte cryopreservation. Germinal vesicle-stage (GV) oocytes and fertilized zygotes have almost exactly the same characteristics. However, zygotes are considerably more resistant to cryo-injuries while GV-stage oocytes are even more sensitive than MII-stage oocytes. Factors that are known to influence their sensitivity include chilling-injury, serious deformation of shape during exposure to and/or removal of cryo-protectants, and hardening of the zona pellucida.

Chilling-injury is probably one of the least understood types of injuries during cryopreservation, involving damage of lipid droplets, lipid-rich membranes, and microtubules. The temperature zone at which such injury occurs is rather high, between +15° (in some biological objects +20°C) and -5°C [12]. The damage to lipids is irreversible and causes death of the oocytes. Compared

to other species, the lipid content of human oocytes is relatively low. Yet, their sensitivity to chilling is still considerable, caused probably by membrane damage and depolymerization of microtubules, with all of the subsequent consequences, including misalignment of chromosomes and aneuploidy [13–17]; however, the latter effect may be less detrimental than earlier supposed [17]. Chilling damage of membranes in human mature oocytes seems to be much more serious than at later developmental stages, e.g. zygotes, a possible cause for the well-known stage-dependent sensitivity [18].

As a result of osmotic effects, serious deformation of the shape may occur when oocytes are exposed to cryo-protectant solutions. However, in spite of the somewhat peculiar morphology that oocytes may exhibit during exposure to cryo-protectants, they do seem to tolerate these deformations rather well. Careful addition of cryo-protectants may minimize deleterious effects of such morphological alterations. An alternative strategy, such as addition of cytoskeleton relaxants used with porcine embryos [19], may not be required in humans. On the other hand, during removal of the cryo-protectant, the spherical shape of the oocyte may allow only a minimal expansion; accordingly, the in-rushing water may disrupt the cell membrane.

Vitrification versus slow freezing

During the past five decades, two major strategies for cryopreservation of oocytes and embryos in mammalian species have been developed [20]. Traditional slow-rate freezing establishes a delicate balance between various sources of injuries, while the principal goal of vitrification is to eliminate ice crystal formation entirely in the whole solution containing the embryos and oocytes. To achieve this ice-free glass-like solidification of solutions, which may also be defined as an extremely increased viscosity, high cryo-protectant concentrations and/or very high cooling rates are required. To decrease the potential osmotic and toxic damage caused by cryo-protectants, recent vitrification methods have focused on increasing the cooling and warming rates [21–24]. Most successful vitrification methods are based on use of extremely small volumes of solution containing the specimens and direct contact between this solution and liquid nitrogen.

One of these approaches, the minimum drop size (MSD) method, was first applied by Arav [25], and further modified by Hamawaki et al. [26]. Based on these earlier results, a novel method, called the ultra-rapid vitrification, was developed for cryopreservation of oocytes and embryos [27]. Ultra-rapid vitrification has been used successfully to cryopreserve oocytes and embryos from a wide variety of mammalian species, and has resulted in a considerable increase in the overall efficiency of cryopreservation of human oocytes and embryos [28–31]. And more recently, based on these huge results and experiences using the ultra-rapid vitrification method for more than two million clinical cases for two decades all over the world, a non-invasive survival vitrification method was established for the standard clinical protocol for human oocytes and embryos (the Cryotec method [9, 32]).

The danger of liquid nitrogen mediated disease transmission

Safety issues regarding open methods of vitrification have been discussed recently in detail [5, 20, 33]. Liquid nitrogen may become contaminated with pathogenic agents and can transmit

these agents to other samples stored in the same tank of liquid nitrogen. Under experimental conditions, transmission has also been demonstrated between embryological samples [34, 35]. Although no disease transmission related to liquid nitrogen-mediated contamination and embryo transfer has been reported for humans or for animals during the past 40 years, a theoretical danger exists and should be minimized with rational measures. According to most observations, hermetical isolation of samples from liquid nitrogen or medium during cooling and thawing considerably decreases cooling and warming rates and, as a consequence, also reduces survival of oocytes. One reasonable solution to this problem is to separate cooling and thawing of oocytes from their storage. Cooling can be performed in liquid nitrogen that is directly provided from the factory, hasn't been in contact with any other biological samples, and has been filtered before use [36, 37]. For storage, samples may be sealed into a pre-cooled, hermetically isolated container, for example 1-mL-diameter cryo-bio-straw (CBS) (IMV, L'Aigle, France). An analogue of the system has been used for OPS vitrification [35] and the required instrument is commercially available (VitSet, Minitüb, Landshut, Germany; [33]). At warming, the end of the 1-mL straw may be cut with sterile scissors while the rest of the straw is still submerged in liquid nitrogen, and the Cryotec can be quickly removed with narrow forceps for immersion into the proper medium. However, high post-warm survival rates of oocytes have not been obtained in these partially closed or fully closed systems, possibly because of the lower cooling and warming rate than those in ultra-rapid vitrification. The fact is that no viral transmission problems have occurred after more than four million cases of clinical applications of the Cryotop and Cryotec method for 20 years in 76 countries. This provides the best practical evidence to indicate the safety of this method with respect to possible liquid nitrogen-mediated disease transmission.

Recent outcomes using ultra-rapid vitrification protocols

The first baby born after human oocyte vitrification was reported by Kuleshova using the OPS method [22, 38] in 1999. However, the survival rate was not high and no replicate results have been reported. This is similar to the first success of human oocyte slow freezing in 1986 [10]. The volume of vitrification solution (VS) is much larger in this method and resulted in a lower cooling/warming rate. Nevertheless, this technique does work well for mammalian embryos, even if less efficiently for oocytes.

In the same year, Kuwayama also obtained the first success of human oocytes vitrification using the ultra-rapid method with an acceptable high survival rate [27]. The protocol of this vitrification has been improved to be simpler and more efficient for everyone's use. The protocol has gradually become used around the world, being adapted for various clinical needs in each country [7, 28, 29].

In Japan, using this method, 91% post-thaw survival rate, 90% fertilization, and 50% blastocysts formation rate after ICSI and *in vitro* culture were first reported [29]. After embryo transfer, a pregnancy rate of 41% was achieved. The ultimate birth rate of those embryos that implanted was 83%. A total of 20 healthy babies were delivered in the clinical trial. This ultra-rapid vitrification method was used to establish the first oocyte bank for unmarried cancer patients in 2001. More than 600 oocytes from 112 patients have been cryopreserved for their future IVF treatment use, and the first delivery was obtained in malignant patients in 2013. Two oocytes of a 16-year-old unmarried malignant student

were vitrified in 2001. They were warmed and ICSI-ET was performed in 2013, and a healthy baby was born in 2014.

In the United States, Katayama et al. [28] repeatedly used ultra-rapid vitrification and achieved a post-warm survival rate for oocytes of 97%, and they obtained the first live baby from a vitrified oocyte in the United States in 2003. They also established the first oocyte bank for unmarried cancer patients and also for healthy women for social reasons in USA in 2002.

In Spain, Cobo et al. [37] reported that the survival of 231 oocytes that were warmed after vitrification was 97%; the respective fertilization, cleavage, and blastocyst rates were 76%, 94%, and 49%. Embryo transfer performed on 23 patients resulted in a 65% pregnancy rate, although with a miscarriage rate of 20%. This Spanish team has used oocyte vitrification for an egg donation program [39]. More than 1000 healthy babies have been born from oocytes that were vitrified by this team alone.

In these two decades, based on huge clinical experiences of ultra-rapid vitrification all over the world, difficulties in the protocol for the embryologists and reasons for lethal damage of the oocytes during the vitrification process have become clear.

And to minimize oocyte loss due to human error for embryologists, improved instrumentation for easier handling has been developed in vitrification and warming-plates. This is because it is a more universal and reliable clinical technique for patients. Regarding VS, there was still lower viscosity of the solutions, difficulty judging completion of the oocyte in VS equilibration, difficulty loading oocytes on vitrification container sheets within a limited time, and stickiness of oocytes to the sheet at warming in thawing solution (TS). But these problems in the method have been solved by the improvement of composition of the solution by the addition of thickening agents, like hydroxy propyl cellulose and xanthan gum [7].

Details of the latest protocol for ultra-rapid vitrification follow.

Latest ultra-rapid vitrification protocol to cryopreserve human oocytes

Timing of vitrification, and ICSI after warming

Oocytes can be vitrified between one and six hours after ovum pick up, and immediately after denudation (cumulus cell removal). ICSI can be performed within two to four hours in culture after the vitrified oocytes have been warmed. This short time of culture is required to allow the oocytes to be recognized as survival and also recover the plasticity of their membranes during the puncture by the ICSI needle.

Vitrification media and container

The media is composed of minimal essential media (MEM), ethylene glycol, and dimethyl sulfoxide as permeable cryo-protectants and Trehalose as non-permeable cryo-protectant. All solutions are serum and protein free.

To obtain the best ultra-rapid cooling and warming rates of the VS containing oocytes, the vitrification container, Cryotec,

consists of a 1.0-mm wide, 1.4-mm long, 0.1-mm thick flexible filmstrip attached to a rigid plastic handle. To protect the filmstrip and the vitrified oocytes on it, a 65-mm long transparent plastic cap is also provided to cover this part during storage in liquid nitrogen (Figure 21.1). The device is sterilized, and should be handled under aseptic conditions and only for one cycle of vitrification.

Working environment and preparation steps

The vitrification procedure has to be performed in a well-ventilated laboratory at room temperature of 25°C to 27°C. Because all equilibration and dilution parameters described here were adjusted for this temperature, it is very important to warm media that have been stored in the refrigerator to 25°C to 27°C. This is easily achieved by placing all the solutions and vials on a clean bench for more than one hour, preferably inside a laminar-flow hood. The only exception is the TS, which should be warmed to 37°C to obtain the highest warming rate of the vitrified oocytes. Note that the basic solution contains Hepes buffer along with bicarbonate buffer, and has been adjusted to maintain the appropriate pH even when exposed to air. Therefore, a carbon dioxide incubator is not required for warming of solutions in closed vials.

Additional tools

Vitrification has to be performed in 300-µL volume three-well plate (Vitri-Plate, REPROLIFE, Japan; Figure 21.2). To obtain the optimum gradual change of osmolality of the extracellular solutions for the best post-warm survival of the oocytes, it is very important that precise proportions of the volume of each solution and transferred solution be used. For practical reasons, a relatively small, thick-walled Styrofoam box (approximately 250 × 150 × 200 cm for length, width, and height) with a minimum of 3-cm thick walls and bottom is suggested, preferably with an appropriate Styrofoam cover. The box should be placed on a stable surface within easy reach but with little risk of accidentally spilling it or pouring off the liquid nitrogen. All safety instructions related to work with liquid nitrogen should be strictly followed. Points for selection of optimal sources and possible pre-treatment of liquid nitrogen will be discussed later. The Styrofoam box should also contain plastic racks for temporary storage of the device.

Ultra-rapid vitrification requires adept handling of oocytes and embryos. For vitrification and warming, a relatively simple stereomicroscope equipped with a zoom lens and capable of providing sharp, contrast images is appropriate. Except for special purposes, there is no need for an upright or inverted compound microscope or for fluorescent equipment. There is no need to restrict illumination if light sources are filtered for UV lights. Use microscope lights only when required.

Equilibration and cooling

Gently mix vials of pre-warmed equilibration solution (ES) and VS (one vial of each). Pour 300 µL of ES into well 1, and 300 µL of VS into wells 2 and 3 in the proper Vitri-Plate (Figure 21.2).



FIGURE 21.1 Cryotec vitrification container, with and without cover cap.



FIGURE 21.2 Vitrification solutions in vitrification plate. Abbreviations: ES, equilibration solution; VS, vitrification solution.

Before starting a vitrification procedure, check the quality and perivitelline space of the oocytes, compare it to the thickness of the zona pellucida, and record any characteristics that might affect oocyte survival. The equilibration and vitrification procedure consist of the following steps.

1. Place the oocytes in the centre of the surface of ES. The oocytes will begin to contract osmotically and they will sink by their own density to the bottom of the well (Figure 21.3).
2. Contraction of the oocytes should occur at the latest within 90 seconds after placing them into the ES. Wait for 12 minutes and observe the recovery of the oocytes. If full re-expansion of oocytes occurs (the perivitelline space should be the same as before equilibration), oocytes should be picked up for the next step. If the volumetric recovery of the oocytes is incomplete, continue the equilibration until 15 minutes all together. The recovery period can be used to prepare the liquid nitrogen container and to label the cryo-containers.
3. Pick up oocytes with the pipette, and expel the oocytes at the middle depth of VS1 with ES. Oocytes will immediately float to the surface of VS1. Expel and wash the inside of the pipette with fresh VS, and pick up the oocytes and expel them again at the bottom of VS. The oocytes will then very slowly float to the middle and stop. When oocytes stop, it is the end of equilibration step, as the weight has become the same inside and outside of the oocytes. Aspirate the oocytes at the tip of pipette to move to VS2 (Figure 21.4).
4. Expel the oocytes into the middle depth of VS2. Expel and aspirate fresh VS from the edge of surface, and expel

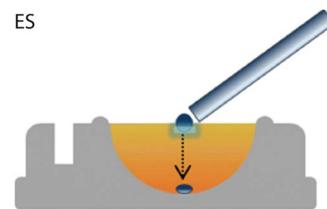


FIGURE 21.3 Equilibration of oocytes in ES. Abbreviation: ES, equilibration solution.

it outside of the well. Aspirate fresh VS2 again from the surface. Blow out VS from the pipette and mix the solution around the oocyte to watch the oocytes to be shrunk from 3D. (Figure 21.5). Expel and wash the inside of the pipette with fresh VS, and aspirate the oocytes at the tip of the pipette to put the oocytes onto the cryo-container set on the slit of the Vitri-Plate.

5. Pick up the oocytes with the pipette in the smallest possible amount of VS and place them on the strip of the cryo-container on the Vitri-Plate near the black triangle mark (Figure 21.6).
6. Immerse the cryo-container directly into the liquid nitrogen in the Styrofoam box and rapidly stir the cryo-container in the liquid nitrogen to obtain the maximum cooling rate (23,000°C/minute). While keeping the cryo-container submerged in liquid nitrogen, cover the strip of the container with the plastic cap using tweezers and then the fingers to ensure it is tightly closed (Figure 21.7).

Warming and dilution of CPAs

An unopened vial of TS and a warming-plate (Figure 21.8) should be pre-warmed to 37°C in an incubator for at least one hour. All other solutions should be kept at room temperature, i.e. 25°C to 27°C.

Gently mix pre-warmed DS and TS vials with an up-and-down movement. Pour 300 µL of DS into well 2, and 1.8 mL of 37°C TS into square well 1 of warming-plate.

The warming and dilution procedures consist of the following steps (dilution is also shown on Figure 21.8):

1. Using tweezers, remove the plastic cap of the cryo-container while it is still submerged in liquid nitrogen. This manipulation can be performed easily if the Styrofoam box is filled almost entirely with liquid nitrogen. The container should be positioned close to the microscope to avoid delay when transferring the cryo-container. The microscope

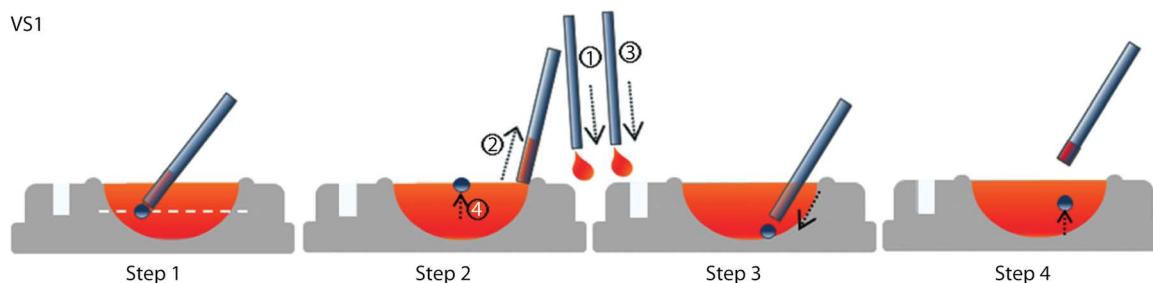


FIGURE 21.4 Equilibration of oocytes in VS1. Abbreviation: VS1, vitrification solution 1.

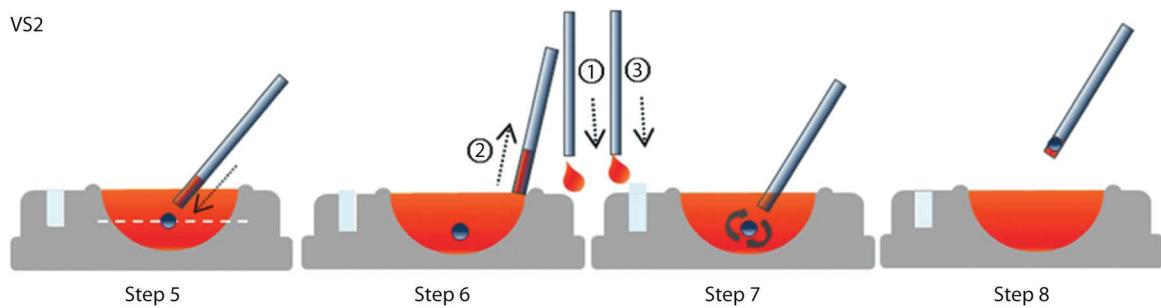


FIGURE 21.5 Confirmation of oocyte shrinkage in VS2. Abbreviation: VS2, vitrification solution 2.

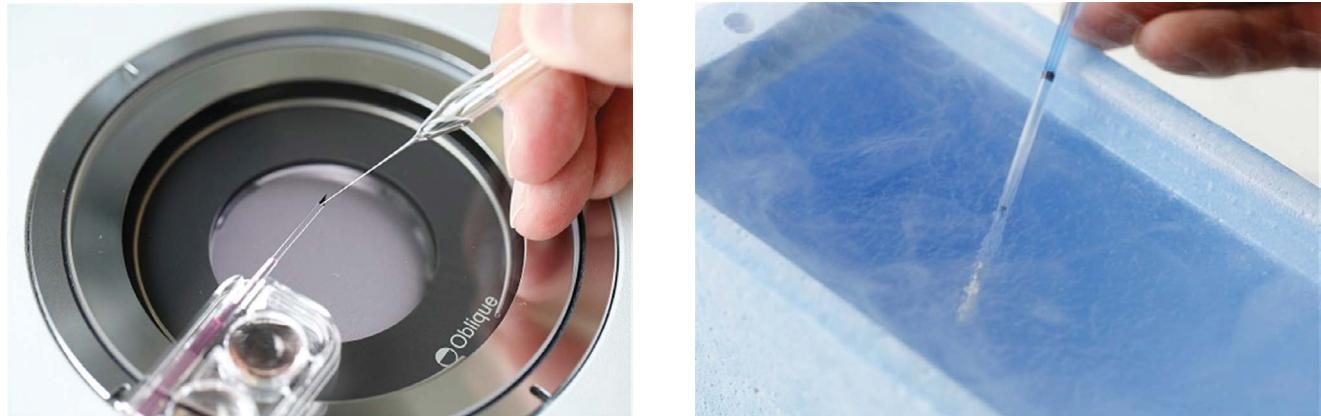


FIGURE 21.6 Easy loading of oocytes to cryo-container using Vitri-Plate.

FIGURE 21.7 Vitrified oocytes (\blacktriangle mark) on cryo-container in LN₂ with tightly closed cover cap.

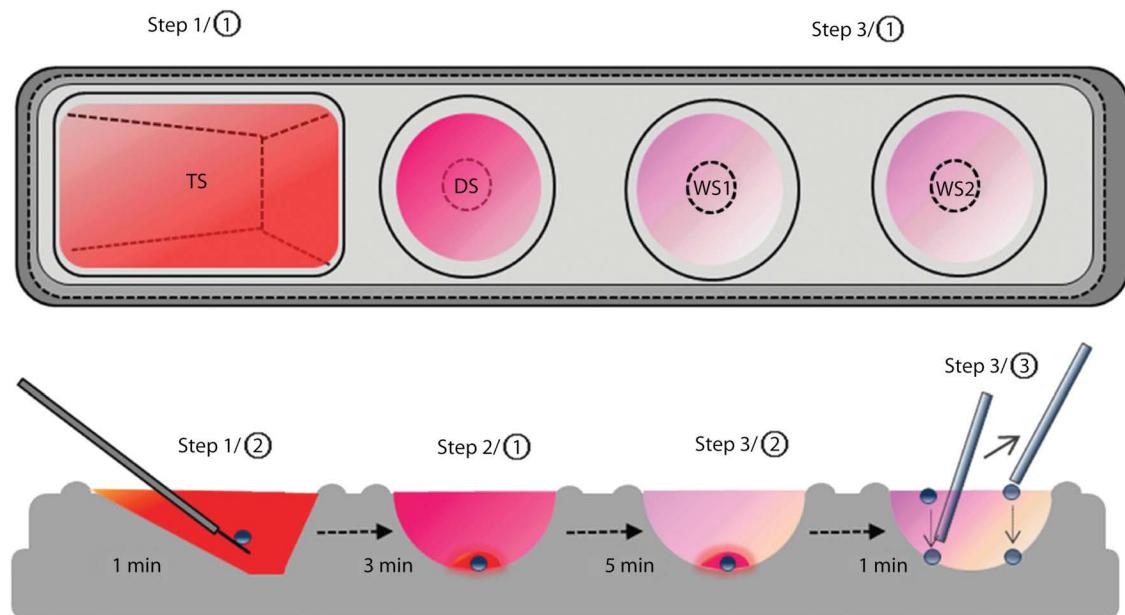


FIGURE 21.8 Warming solutions in warming plate and warming procedure. Abbreviations: DS, dilution solution; TS, thawing solution; WS1 washing solution 1; WS2, washing solution 2.

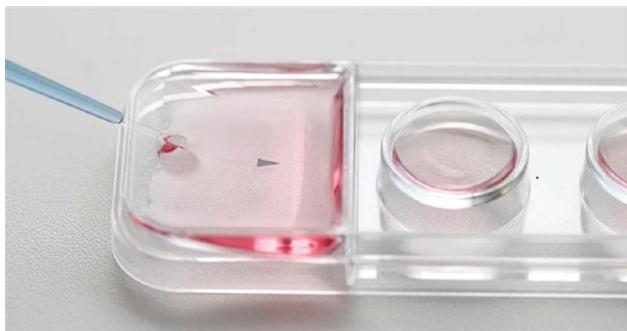


FIGURE 21.9 Warming: oocyte on cryo-container sheet in thawing solution in the warming-plate.

- should be focused at the centre of the TS of the warming-plate with low magnification.
2. Hold the cryo-container and look for the black mark while maintaining the tip submerged in liquid nitrogen. Remove the cryo-container with a rapid movement from the liquid nitrogen and place the tip immediately into the middle of the square TS well of the warming plate (Figure 21.9).
 3. Find the oocytes by adjusting the focus on the cryo-container sheet. One minute after immersing into TS, while keeping the sheet in the middle of TS, oocytes will separate themselves from the sheet and will begin to float. Follow all movements of the oocytes continuously, as they become transparent at this phase of the procedure and it is easy to lose them. Later, they will regain their normal appearance.
 4. Gently pick up the oocytes into the pipette and aspirate an additional 3-mm-long TS column to the tip of the pipette. Transfer the pipette to the bottom of DS well and expel the contents gently to the centre of the bottom (deepest place): first the TS media, allowing it to form a small “mountain” of fluid, then the oocytes to the bottom of this mountain. Just do nothing. Wait for three minutes (Figure 21.10).
 5. Subsequently, the same method of transfer should be applied but with different solutions: oocytes will be placed to the bottom of the mountain formed from DS medium in the WS1 dish for five minutes, without any stirring or mixing of the media.
 6. Place oocytes onto the surface of WS2 and wait for one minute.
 7. Finally, oocytes are transferred into the culture dish and their morphology is examined under the stereomicroscope. ICSI can be performed after a recovery period of at least one hour.

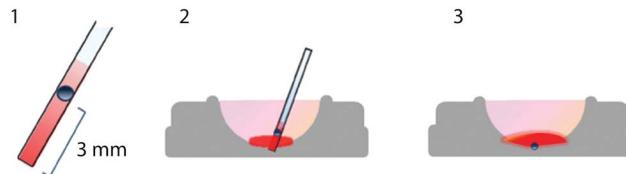


FIGURE 21.10 Gradual replacement of the solutions (thawing solution to dilution solution/dilution solution to washing solution).

High survival of human oocytes by ultra-rapid vitrification

Before being cryopreserved, the potential development rate of oocytes is 100%. If some oocytes undergo serious damage during cryopreservation, those oocytes die, resulting in a lower overall survival rate. A lower survival rate is the evidence of increasing damage caused by cryopreservation. Therefore, especially in clinical applications of vitrification, it is very important that the highest survival be attained not only for the efficiency of the treatment but also to ensure the likelihood of producing normal, healthy babies. Such high post-warming survival of oocytes can be obtained using an ultra-rapid vitrification method.

As a result of personal communication with colleagues in more than 76 countries, I estimate that more than 100,000 oocytes have been vitrified by the ultra-rapid method, and in most of the centres, the recent survival rate of vitrified oocytes after warming using the latest ultra-rapid vitrification protocol is almost 100%, and more than 10,000 healthy babies have been delivered thus far. The fact that such results have been reported by this many independent clinical groups in different countries with no direct or commercial connection for the past 10 years may indicate that a reliable clinical procedure to cryopreserve human oocytes has been established.

Conclusion

Cryopreservation of oocytes is regarded as one of the most demanding tasks of human-assisted reproduction. With scrupulous attention to numerous details and proper application of the latest vitrification techniques, efficiency of the procedure has been substantially improved.

The latest vitrification method has resulted in almost 100% survival rate followed by excellent fertilization, blastocysts development, pregnancy, and births after ETs, comparable to those achieved with non-vitrified control oocytes.

The technique can be useful in diverse situations where oocyte storage is required or considered.

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References

1. Polge C, Smith AY, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature (London). 1949;164:666.
2. Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos frozen to -196°C and -269°C. Science. 1972;178:411–4.
3. Wilmut I. The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. Life Sci. 1972;11:1071–9.
4. Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing, and transfer of an eight-cell embryo. Nature. 1983;305:707–9.
5. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The cryotop method. Theriogenology. 2007;67:73–80.
6. Kuwayama M, Cobo A, Vajta G. Vitrification of oocytes: General considerations and the use of the cryotop method. In: Vitrification in Assisted Reproduction. Tucker MJ, Liebermann J (eds.). London: Informa Healthcare, pp. 119–128, 2008.

7. Gandhi G, Ramesh S, Khatoon A. Vitrification of oocytes: General considerations. In: Vitrification in Assisted Reproduction. UK Springer. pp. 17–30, 2014.
8. Kuwayama M, Gandhi G, Kagalwala S, Ramani R. Vitrification: An overview. In: Vitrification in Assisted Reproduction. UK Springer, pp. 1–7, 2014.
9. Kuwayama M, Gandhi G, Kagalwala S, Khatoon A. Oocyte Banking: Current perspectives. In: Vitrification in Assisted Reproduction. UK Springer. pp. 89–95, 2014.
10. Chen C. Pregnancy after human oocyte cryopreservation. *Lancet*. 1986;1(8486):884–6.
11. Liebermann J, Tucker MJ. Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertil Steril*. 2006;86:20–6.
12. Leibo SP, Martino A, Kobayashi S, Pollard JW. Stage-dependent sensitivity of oocytes and embryos to low temperatures. *Anim Reprod Sci*. 1996;42:45–53.
13. Magistrini M, Szollosi D. Effects of cold and of isopropyl N-phenylcarbamate on the second meiotic spindle of mouse oocytes. *Eur J Cell Biol*. 1980;22:699–707.
14. Sathananthan AH, Ng SC, Trounson AO, Bongso A, Ratnam SS, Ho J, Mok H, Lee MN. The effects of ultrarapid freezing on meiotic and mitotic spindles of oocytes and embryos. *Gam Res*. 1998;21:385–401.
15. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril*. 1990;54:102–8.
16. Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: New perspectives regarding oocyte survival. *Hum Reprod*. 2001;16:411–6.
17. Stachecki JJ, Munne S, Cohen J. Spindle organization after cryopreservation of mouse, human, and bovine oocytes. *Reprod Biomed Online*. 2004;8:664–72.
18. Ghetler Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Hum Reprod*. 2005;20:3385–9.
19. Dobrinsky JR, Pursel VG, Long CR, Johnson LA. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. *Biol Reprod*. 2000;62:564–70.
20. Vajta G, Nagy PZ. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online*. 2006;12:779–796.
21. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol Reprod*. 1996;54:1059–69.
22. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev*. 1998b;51:53–58.
23. Lane M, Schoolcraft WB, Gardner DK. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril*. 1999;72:1073–8.
24. Lane M, Bavister BD, Lyons EA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. *Nat Biotechnol*. 2001;17:1234–6.
25. Arav A. Vitrification of oocytes and embryos. In: New Trends in Embryo Transfer. Lauria A, Gandolfi F (eds.). Cambridge UK: Portland Press. pp. 255–64, 1992.
26. Hamawaki A, Kuwayama M, Hamano S. Minimum volume cooling method for bovine blastocyst vitrification. *Theriogenology*. 1999;51:165.
27. Kuwayama M, Kato O. All-round vitrification method for human oocytes and embryos. *J Assist Reprod Genet*. 2000;17:477.
28. Katayama P, Stehlik J, Kuwayama M, Kato O, Stehlik E. High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertil Steril*. 2003;80:223–4.
29. Kuwayama M, Vajta G, Kato O, Leibo S. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online*. 2005;11:300–8.
30. Kuwayama M, Vajta G, Ieda S, Kato O. Vitrification of human embryos using the CryoTip™ method. *Reprod Biomed Online*. 2005b;11:608–14.
31. Kuwayama M, Leibo S. Cryopreservation of human embryos and oocytes. *J Mamm Ova Res*. 2010;25:79–86.
32. Dalvit G. Vitrification of Day 2–3 human embryos using various methods. In: Vitrification in Assisted Reproduction. UK Springer. pp. 65–70, 2014.
33. Vajta G, Kuwayama M, Vanderzwalmen P. Disadvantages and benefits of vitrification. In: Vitrification in Assisted Reproduction. Tucker MJ, Liebermann J (eds.). London: Informa Healthcare. pp. 33–44, 2008.
34. Bielanski A, Nadin-Davis S, Sapp T, Lutze-Wallace C. Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology*. 2000;40:110–6.
35. Bielanski A, Bergeron H, Lau PC, Devenish J. Microbial contamination of embryos and semen during long-term banking in liquid nitrogen. *Cryobiology*. 2003;46:146–52.
36. Vajta G, Lewis IM, Kuwayama M, Greve T, Callesen H. Sterile application of the open pulled straw (OPS) vitrification method. *Cryo-Letters*. 1998;19:389–92.
37. Cobo A, Kuwayama M, Pérez S, Ruiz A, Pellicer A, Remohí J. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the cryotop method. *Fertil Steril*. 2008;89:1657–64.
38. Kuleshova L, Gianaroli L, Magli C, Trounson A. Birth following vitrification of a small number of human oocytes. *Hum Reprod*. 1999;14:3077–9.
39. Cobo A, Remohí J, Chang CC, Nagy ZP. Oocyte cryopreservation for donor egg banking. *Reprod Biomed Online*. 2011;11:300–8.

22

THE HUMAN EMBRYO

Vitrification

Zsolt Peter Nagy and Ching-Chien Chang

Introduction

Decades ago, most assisted reproductive technologies including *in vitro* fertilization (IVF) and cryopreservation of embryos by traditional freezing were applied to humans almost immediately after the first successes in some experimental or domestic species. However, there are some techniques, where efforts to adopt a new approach were insufficient and sporadic, consequently the practical application has been considerably delayed. Vitrification belongs to this group. Reasons for this delay may include the fact that slow-freezing/thawing of zygote-, cleavage-, and blastocyst-stage human embryos was somewhat efficient, providing a “reasonable” survival rate of embryos (somewhere between the 50% to 80% range). At the same time, vitrification seemed “scary,” requiring the use of very high concentrations of cryo-protectants, and very precise handling/timing, while it also seemed “less sophisticated” because it relies fully on manual technology compared to slow-freezing where automatic traditional freezers are used. High concentrations of cryo-protectants required for vitrification discouraged some potential users initially. Finally, none of the major suppliers were eager to replace their expensive freezing machines with a much simpler system required for vitrification (though, very recently there have been some efforts to develop an instrument that would allow a “semi-automated” vitrification process) [1, 2].

Some scientists in the early 2000s started moving from domestic animal embryology to the human field to apply the technique of vitrification. However, additional years were still required to get the approach acknowledged, to develop commercially available tools and kits, and to teach both distributors and consumers about the benefit of vitrification. Eventually, the overwhelming comparative evidence made clear to almost everybody that in all developmental stages, vitrification produces significantly better survival and more competent oocytes/embryos than traditional freezing. Today, the rapidly increasing interest towards vitrification creates novel problems such as diversity of tools and media, lack of information regarding ingredients, inconsistency in teaching and application. Legal concerns on biosafety issues have also emerged, although no scientific proof exists for the magnitude or existence of any risks.

In this chapter, we summarize the basic features of vitrification, explain some special aspects of vitrification, and provide data about the efficiency of vitrification for cryopreservation of human pre-implantation stage embryos at different developmental stages. Also discussed is how the highly efficient vitrification (both embryo and oocyte) method has contributed to a paradigm shift in how assisted reproduction treatment is practiced today. The effect of vitrification is clearly demonstrated by the dramatic increase of cryopreservation cycles (“freeze all” IVF cycles) in the United States. Based on the Center for Disease Control (CDC) data, in 2007 there were only 2020 “freeze all” cycles, in 2013

there were 27,564, and in 2019 (the most recent available data) there were 121,086 freeze all cycles (Figure 22.1).

For terms and definitions, we accept and use the excellent review and suggestions of Shaw and Jones [3]. For the basic principles of cryobiology we refer to earlier reviews [4–7].

Main cryopreservation approaches

Within approximately a decade after the first successes with cryopreservation of mammalian embryos [8–12], the first human pregnancies were achieved [13, 14]. All these works were performed with traditional slow freezing. Vitrification was first applied for cryopreservation of mammalian embryos in 1985 [15] but regarded as a curiosity and experimental procedure for almost a decade, when practical application was started in domestic animal embryology and sporadic approaches in humans. Competitive vitrification strategies for human embryo and oocyte cryopreservation have only been developed 15–18 years ago (in the mid-2000s).

The strategies of the two approaches are basically different. Far the most important source of damage at cryopreservation is ice crystal formation. To minimize this injury, application of various chemicals (cryo-protectants) is required, which, unfortunately, may also induce various injuries including toxic and osmotic damage.

Just to “recap,” the mostly “retired” traditional slow-rate freezing creates a delicate balance between these factors. Embryos are typically exposed to 1–2-mol/L solutions of permeable and (less concentrated, if any) non-permeable cryo-protectants, then loaded into a 0.25-mL straw, sealed and cooled to –6°C relatively rapidly, by placing the straws into a controlled-rate freezer. With the given cryo-protectant concentration, no spontaneous ice formation occurs at this temperature; however, ice nucleation can be induced by “seeding,” i.e. touching the straw with a forceps that has been previously immersed into liquid nitrogen. The controlled-rate freezer is adjusted to make a very slow cooling (usually 0.3°C/minute, to around –30°C), then the straws are immersed into liquid nitrogen for a final cooling and storage. The slow rate of cooling allows solution exchange between the extracellular and intracellular fluids without serious osmotic effects and deformation of the cells (this fact is reflected in the other name of the procedure: equilibrium freezing; [16]).

The strategy of vitrification is much more radical. The main purpose (according to the cryobiological definition) is the complete elimination of ice formation in the whole solution the sample is cooled in.

Evidently, this can only be performed with the use of high cryo-protectant concentration, which may theoretically induce serious toxic and osmotic damages. A huge variety of cryo-protectants were tested in different studies along with many “technical approaches” (see Table 22.1), and as a result, most

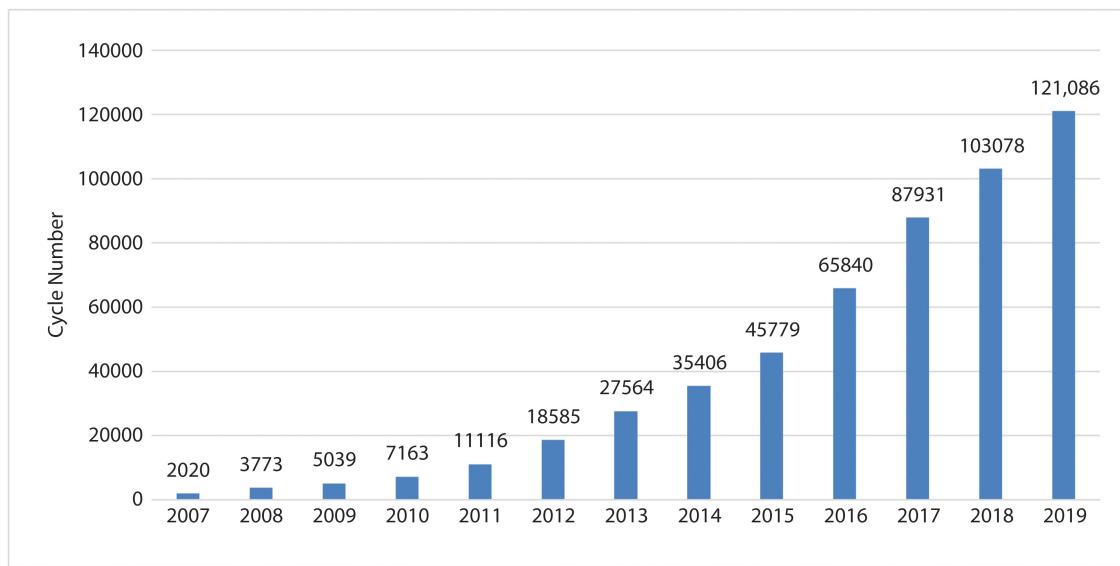


FIGURE 22.1 The impact of vitrification on the number of IVF cycles with “freeze all” in the United States, from 2007 to 2019.

TABLE 22.1 Various Vitrification Techniques in Embryology

System	Reference
Direct dropping into liquid nitrogen	Landa and Tepla [76]
Electron microscopic grids	Martino et al. [25]
Open-pulled straw (OPS)	Vajta et al. [83]
Glass micropipettes (GMP)	Kong et al. [239]
Super-finely pulled OPS (SOPs)	Isachenko et al. [240]
Gel-loading tips	Tominaga and Hamada [241]
Sterile stripper tip	Kuleshova and Lopata [125]
Flexipet denuding pipette (FDP)	Liebermann et al. [242]
Fine-diameter plastic micropipette	Cremades et al. [243]
100 µl pipetting tip	Hredzak et al. [244]
Closed-pulled straw (CPS)	Chen et al. [245]
Sealed open-pulled straws	Lopez-Bejar and Lopez-Gatius [246]
Cryotip	Kuwayama et al. [69]
Cryoloop	Lane et al. [91]
Nylon mesh	Matsumoto et al. [97]
Minimum drop size (MDS)	Arav [247]
Minimum volume cooling (MVC)	Hamawaki et al. [99]
Hemi-straw system (HSS)	Vanderzwalmen et al. [100]
Cryotop	Kuwayama et al. [68]
Vitmaster	Arav et al. [85]
Solid surface vitrification (SSV)	Dinnyes et al. [113]

Source: Reprinted from Vajta and Nagy [248] with permission from Reproductive Healthcare Ltd.

vitrification-based kits have very similar composition (similar components and concentrations) and similar techniques/handling. Cell shrinkage caused by non-permeable cryo-protectants, and the incomplete penetration of permeable components may cause a relative increase of intracellular concentration of macromolecules that is enough to hamper intracellular ice formation. Accordingly, vitrification belongs to the group of non-equilibrium cryopreservation methods.

Another possibility to minimize the chance of ice formation during vitrification is to increase the cooling and warming rates. The higher the cooling rate, the lower the required cryo-protectant concentration is, and vice versa. Eventually, even the radical approach of vitrification has to establish a delicate balance, as it requires (i) establishment of a safe system for maximal and reliable cooling (and warming) rates while avoiding consequent damage including fracture of the zona pellucida or the cells, and (ii) elimination or minimization of the toxic and osmotic effects of high cryo-protectant concentrations needed to obtain and maintain the glass-like solidification.

There is, however, a small, poorly defined group of cryopreservation techniques that shares some features with both vitrification and slow rate freezing. In this method, cryo-protectant concentrations are insufficient to establish vitrification [9, 17–19]. This approach has been established entirely empirically, and does not meet any supposed requirements of cryopreservation in embryology. Although ice is formed in the solution, under certain (and sometimes unpredictable) conditions embryos survive and develop further [20, 21]. However, the lack of control may result in inconsistent survival and developmental rates. On the other hand, some of the early experiments characterized as rapid freezing were in fact vitrifications [22, 23].

Injury and prevention during cryopreservation

Exposition to deep sub-zero temperatures is a situation mammalian cells never meet under physiological circumstances. The injury may occur at all phases of the procedure.

During cooling, different types of damage may occur when embryos pass through three overlapping temperature zones.

At relatively high temperatures, between +15 and –5°C, the chilling injury is the major factor, damaging predominantly the cytoplasmic lipid droplets and microtubules including the meiotic spindle [24–26]. While the latter damage may be reversible, the former is always irreversible and contributes to the death of cryopreserved lipid-rich oocytes and embryos of some species.

Between -5 and -80°C , extracellular or, predominantly, intracellular ice crystal formation is the main source of injury.

Temperatures between -50 and -150°C can cause fracture damage to the zona pellucida or the cytoplasm [27] are postulated to occur (although the mechanism and the actual temperature of occurrence is not entirely defined). However, it is unlikely that zona fracture could occur as a simple consequence of osmotic stress, as suggested by Smith and Silva [6].

Storage below -150°C (typically in liquid nitrogen, at -196°C) is probably the least dangerous phase of the cryopreservation procedure.

Importantly, accidental warming is probably the most frequent form of injury, which definitely puts vitrified samples at risk if not handled appropriately [28]. The effect of background irradiation seems to be less harmful than supposed, and is not a significant source for DNA injury in a realistic time interval, i.e. years, decades, or even centuries [29]. There is increasing concern regarding possible disease transmission between the stored samples mediated by the liquid nitrogen—even though there are no reported cases in literature involving embryos.

At warming, the same types of injuries may occur as at cooling, obviously in inverse order. One of the most likely reasons for injury is recrystallization during warming, which nearly always occurs. To avoid or minimize its potential damage, the addition of certain components to the cryo-solution has been investigated, as well as adjusting the speed of warming (relative to the speed of cooling) [30, 31].

Apart from these processes, there are some partially understood injuries including damage of intracellular organelles, cytoskeleton, and cell-to-cell contacts [32, 33].

All embryos subject to cryopreservation may suffer considerable damage during cooling and warming. Fortunately, they also have a remarkable, sometimes surprising ability to repair fully or partially this damage, and in the best case to continue normal development. All cryopreservation methods try to decrease the damage and facilitate the regeneration process.

Cryo-protectants are a diverse group of simple or complex, permeable or non-permeable, organic or inorganic compounds with two common features: they are water-soluble and they protect the cells from cryo-injuries. The range is wide, expanding from well-known simple organic solvents such as ethanol to the complex, partially known substances such as serum or egg yolk. Permeable cryo-protectants enter the cells and minimize ice formation with various mechanisms depending on their structure and chemical activity, whereas non-permeable cryo-protectants remain outside the cells and minimize ice formation by removing water from the cells by osmotic effect. However, there are certain overlaps between the two groups, especially in vitrification methods, where the usually applied short exposition to the concentrated, theoretically permeable components do not allow full equilibrium, therefore part of the effect of permeable cryo-protectants is dehydration, as well. Additionally, both permeable and non-permeable components may have some other specific cryo-protectant effects, for example, stabilization of cell membranes, the meiotic spindle, or other cellular structures [34]. Unfortunately, most cryo-protectants have some negative effects, including toxicity and, obviously, osmotic effect. Toxicity is usually in direct correlation with the concentration of the substance, the temperature, and the time of exposure. The osmotic effect is mostly proportional to the concentration. In case of permeable or partially permeable cryo-protectants, the osmotic effect can be minimized by

slow, stepwise addition and removal during equilibration and dilution, respectively. The mechanism and reasons for damage during cryopreservation as well as the precise protective mechanisms of cryo-protectants are poorly understood at present. The effects of a given cryo-protectant may substantially differ at physiological and low temperatures; thus, the retrospective analysis of damage may result in faulty conclusions. Considering these uncertainties, it is not surprising that the vast majority of existing cryopreservation techniques were established empirically, based on rough morphological changes observed under a stereomicroscope, and have been justified by the outcome, i.e. *in vitro* and *in vivo* survival. It is more recent development that using highly sophisticated diagnostics would help to assess freezing conditions, such as using protein expression to detect gene expression [35, 36].

Vitrification

Cryo-protectants

No cryo-protectants exclusively designed or used for vitrification have been developed yet. Certain components and combinations (for example ethylene glycol, DMSO, and sucrose) are typically used for vitrification purposes, and the concentration of specific components is significantly higher at vitrification than in traditional or rapid freezing.

The most common permeable components are ethylene glycol, propylene glycol, acetamide, glycerol, raffinose, and dimethyl sulfoxide (DMSO), and these have been tested in various combinations [5, 37]. Due to low toxicity, high permeability, and excellent ice-blocking ability, ethylene glycol is an almost indispensable component of all cryo-protectant solutions. However, a common strategy to decrease the specific toxicity of any one cryo-protectant is to use the mixture of two permeable cryo-protectants, i.e. a mixture of ethylene glycol and either DMSO, propylene glycol, or, less typically other components. Eventually the mixture of ethylene glycol and DMSO appears to be used frequently [38, 39]. According to some studies, the permeability of this mixture is higher than that of the individual components [40]. It should be noted that the earlier concerns regarding the genotoxicity and cytotoxicity of DMSO have been dismissed [41, 42].

Commonly used non-permeable cryo-protectants include mono- and disaccharides, sucrose, trehalose, glucose, and galactose [43–45]. Recently, sucrose has become almost a standard component of vitrification mixtures. This is true even though nearly all comparative investigations proved the superiority of trehalose. Sucrose along with other sugars may not have any toxic effects at low temperatures, but may compromise embryo survival when applied extensively to counterbalance embryo swelling after warming [46–48], although this effect was not always demonstrated [49]. Several polymers were also suggested for the purpose, including polyvinylpyrrolidone, polyethylene glycol, Ficoll, dextran, and polyvinyl alcohol [50–55]. However, from this group the only widely used compound is Ficoll, predominantly in combination with ethylene glycol and sucrose [56]. Various forms of protein supplementation have also been used, including egg yolk, but its optically dense appearance made the microscopic manipulation rather difficult. High concentrations of sera of different origin as well as serum albumin preparations [57] are common additives. In the bovine model, recombinant albumin and hyaluronan were also effective [58]. On the other hand, the use of antifreeze proteins isolated from arctic animals [59–61] has

largely been abandoned. More recently, hydroxypropyl cellulose (HPC) was investigated as a replacement for serum-derived protein for use in cryo-protectant solutions, and results have been promising [62, 63].

Another practical feature is the stepwise addition of increasing concentrations of cryo-protectants [57, 64–66]. After several early attempts, the two-step equilibration has become the most commonly used approach, with the first solution containing approximately 50% of the final cryo-protectant concentration. Embryos and oocytes are equilibrated for a relatively long period (5–15, sometimes up to 21 minutes) in the first solution, then for a short period (approximately 1 minute) in the second one [67–69]. This approach may increase the toxic effect slightly, but provides a much better protection for the whole cell, and may be especially beneficial in the case of large substances with a low surface/volume ratios, including oocytes or early-stage embryos. On the other hand, earlier attempts to cool the concentrated solution to 4°C to decrease toxicity have been found later to be unnecessary. Because of the much higher concentrations of cryo-protectants (CPAs) used in vitrification, it was initially assumed that intracellular concentrations of CPAs are higher after vitrification than after slow-freezing—giving concerns on the toxicity of these CPAs. However, in a recent, elegant study, it was demonstrated that intracellular concentration of cryo-protectants are actually lower after vitrification than after slow freezing, despite exposure to higher concentration of cryo-protectant solutions [70]. Very recently, there are attempts to use microfluidic technique to provide a more controlled and gradual increase of cryo-protectants to the vicinity of the embryo (or oocyte) using robotic systems to improve outcomes [71].

Tools of cryopreservation used for vitrification

Plastic insemination straws or cryovials were used initially for vitrification experiments. These tools were not designed for the special purpose of vitrification, had a thick wall, and required a relatively large amount of solution for safe loading. Accordingly, the cooling and warming rates were quite limited (approximately 2500°C/minute for straws [72]; and even less for cryovials). This relatively low rate was still hazardous to perform, as direct immersion into liquid nitrogen at cooling, and transfer to a water bath at warming, induced extreme pressure changes in the closed system and frequently led to the collapse or explosion of the straws and loss of the sample. One of the other consequences of these manipulations was the decreased and inconsistent rates: the temperature of the vapour of liquid nitrogen is variable, depending on many factors, and the definition of “room temperature” laboratory air may mean 5–7°C differences, even at the same place on the same day. Consequently, a minimum 5–7-mol/L cryo-protectant concentration was required, and chilling injury could not be lowered to the level occurring at slow freezing.

Some scientists have investigated the use of an instrument, called a VitMaster, to achieve higher cooling rates. (VitMaster is able to lower the temperature of liquid nitrogen from its boiling point of -196°C to around -208°C—applying vacuum—thus the nitrogen then changes from its liquid state to a slush, which prevents an insulating pocket of gas forming around the sample, resulting in faster cooling). Although outcomes of vitrification using VitMaster tended to be somewhat better than “traditional” vitrification [73, 74], its use has not become part of the daily routine. More recently, efforts were made to develop an instrument

that can offer some level of automation for vitrifying samples [1]. This “Gavi” system can automatically perform equilibration steps before closed vitrification is performed for embryos. The warming, however, has to be performed manually, and currently the system is not proven to perform equivalently for oocyte cryopreservation. Although technically challenging, there are continued efforts to create an automatic (or semi-automatic) vitrification device [75].

Increasing cooling rates with new carrier tools

Although the increased cooling and warming rate was a well-known way to keep the concentration of cryo-protectants as low as possible, and minimize the related toxic and osmotic injuries, this option has remained unexploited for a relatively long period of time. The first purpose-made tools were only produced approximately 20–25 years ago. Today, however, there is a huge variety of tools, methods, and approaches available, and without adequate comparative studies, the selection of the best choice is a serious problem for embryologists working in a routine human IVF laboratory.

The most logical way to increase cooling and warming rates is to decrease the volume of the solution that surrounds the sample, and to establish a direct contact between the sample and the liquid nitrogen.

Seemingly the simplest way to accomplish this task is the direct dropping into liquid nitrogen [67, 76–78]. Unfortunately, to form a drop from a water-based solution requires a relatively large amount of solution (4–6 µL), and the drop does not sink immediately into the liquid nitrogen, because for the initial seconds the drop is surrounded by the vapour that is induced by the warm solution, and does not allow the sample to sink (see Table 22.1 for the different approaches investigated).

Accordingly, some carrier tools have been used to push the sample immediately below the level of liquid nitrogen, to serve as a storage device after cooling, and to facilitate quick warming as well. Electron microscopic grids used for this completely different purpose have proved the practical value of the idea first [76, 77, 79, 80]. In this system, the size of the drop surrounding the sample was extremely small, as after loading, most of it was removed by placing the grid on a filter membrane. The thermocductive metal grid also contributed to improving the cooling and warming rates. Surprisingly, the solidified cryo-protectant solution fixed the sample safely to the grid during cooling and storage, and released it easily after warming [81]. However, the storage and handling of the tiny grid has been a demanding task.

The first purpose-made tool for vitrification was the open pulled straw (OPS), a modification of a standard 0.25-mL plastic straw, with decreased diameter and wall thickness. This modification enabled loading with the capillary effect, and the minimum volume decreased to approximately 0.5–1 µL, i.e. five to ten times smaller than that of the original straw, which results in approximately a tenfold increase in the achievable cooling and warming rates, and 30% decrease of the cryo-protectant concentration required for vitrification. The OPS has become the most widely used approach for ultra-rapid vitrification [82–90].

The cryoloop was another approach applied earlier using the small volume–direct contact principle. It consists of a small nylon loop attached to a holder and equipped with a container (Figure 22.2). It has been used for cryopreservation in crystallography and is now used widely for oocyte and embryo cryopreservation [91–94]. The solution film bridging the hole of the

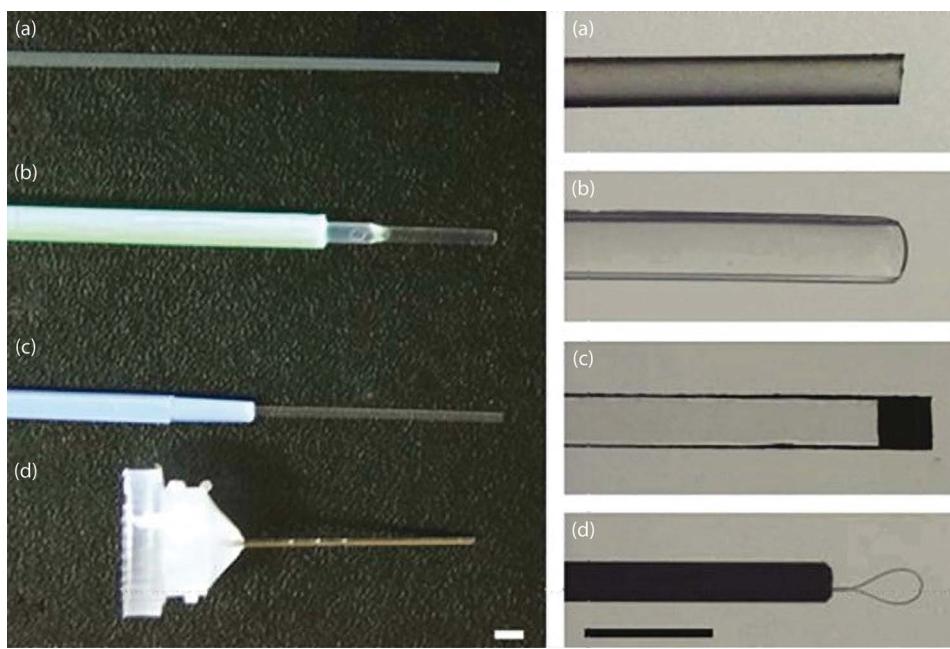


FIGURE 22.2 Examples for commercially available tools used as carriers for high-speed vitrification. (a) Open pulled straw, OPS (Minitüb, Landshut, Germany); (b) McGill Cryoleaf (MediCult, Jyllinge, Denmark); (c) Cryotop (Kitazato, Tokyo, Japan); (d) Cryoloop (Hampton Research, Aliso Viejo, California, United States). Bars represent 2 mm.

loop is strong enough to hold the oocyte or the embryo, and with this minimal solution volume, the achievable cooling rate may be extremely high, up to an estimated $700,000^{\circ}\text{C}/\text{minute}$ [95]. Using this tool, safe cryopreservation can be achieved even in the vapour of liquid nitrogen [96, 97].

The minimum drop size (MDS) method of Arav [98] consists of a small droplet of vitrification solution containing the oocyte or embryo placed on a solid surface that is immersed into liquid nitrogen. The approach was used later with some modifications called the minimum volume cooling (MVC; [99]) or in the hemi-straw system (HSS; [100]), where the carrier tool was a cut-open straw.

Currently, the most commonly used tool for the vitrification of human oocytes and embryos is the Cryotop, an advanced version of the MVC technology [69, 99]. It consists of a flexible transparent plastic film attached to a handle, and also equipped with a protective tube to avoid damage to the film during storage in liquid nitrogen. The sample is loaded on the film, the excess solution is removed, and the film is immersed into the liquid nitrogen. At warming, the Cryotop is quickly removed from the liquid nitrogen, and the film is immersed into the warming medium. Since its first introduction, a good number of studies confirmed its value [101–103]. Yet other carriers, such as Cryolock, and Cryotec, similar in its design to Cryotop, are gaining more popularity and being used efficiently [104, 105].

Cryopette is probably the first carrier that is designed to combine the benefit of very low volume solution with the advantage of a closed system. There are other closed carriers that have been investigated and tried for the use of embryo vitrification, including the Rapid-I [106, 107] and the CBS-VIT High Security (HS) straw, demonstrating satisfactory outcomes [108, 109]. Based on published studies, it appears that closed systems are also able to

provide adequate outcomes for embryo vitrification, however, open systems are more likely to provide superior results when oocytes are vitrified and to preserve the original physiological cell condition [110, 111].

The flow chart of a typical high-speed vitrification procedure is summarized in Figure 22.3.

Decreased vapour formation for increased cooling rates

One major limitation of the achievable cooling rates of the sample is the vapour that is formed around it at immersion. At -196°C , liquid nitrogen is at boiling point. Accordingly, a submerged warmer item will induce an extensive evaporation around the sample, producing a thermo-insulating coat around the sample and decreasing the achievable cooling rate, especially at the initial moments, when the chilling injury may develop.

One possibility to avoid this phenomenon is to expose liquid nitrogen to a vacuum for several minutes. Part of the liquid nitrogen will evaporate, and the rest will cool down to -203 to -207°C , where it starts to get solidified, i.e. slush is formed. As the nitrogen escapes from the fragile boiling zone, the immersed sample creates a minimal evaporation, consequently the cooling rate gets considerably higher [85, 86, 112]. While using “super-cooled” LN₂ offers the aforementioned theoretical benefits, the use of VitMaster (and this process) has not gained much application in real life. Probably because it is possible to achieve excellent outcomes without this approach, and also because the use of this instrument is not very practical.

The other way to eliminate the vapour is the use of pre-cooled metal surfaces instead of liquid nitrogen for cooling. It can be performed by immersing a metal block into liquid nitrogen [113], or by using a more sophisticated, commercially available version (CMV; Cryologic, Australia).

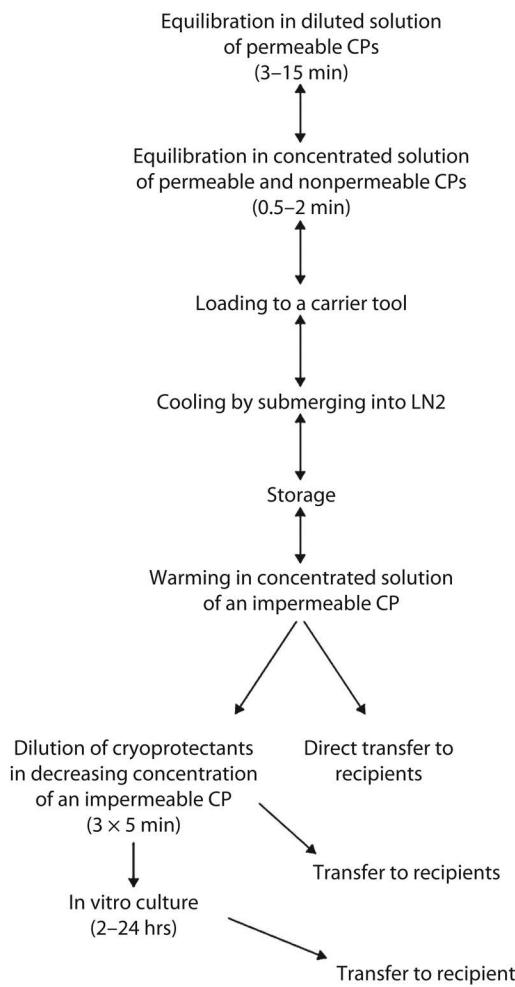


FIGURE 22.3 Flow chart of a typical high-speed vitrification procedure.

The available free comparative data do not provide entirely convincing evidence regarding the superiority of these vapour-minimizing or vapour-free approaches compared to other vitrification procedures.

Transmission of infectious agents

One of the concerns regarding the use of vitrification in human embryology is the potential risk of liquid nitrogen-mediated disease transmission. To understand better, we need to consider the following:

1. Semen and oocyte collection, processing/handling and cryopreservation protocols are not sterile procedures [114]; consequently, the contents of virtually all stored straws and cryovials may be a source of infection.
2. In human embryology, liquid nitrogen may also be contaminated by the surface of straws, cryovials, racks, and other tools that are usually not handled fully aseptically. Accordingly, the presence of infective agents is not strictly related to leaky or open containers.
3. Seemingly sterile containers may not be as safe as supposed. Infection may occur in common straws in slow

freezing (through the holes of incomplete sealing, or pores of the plastic walls), and most cryovials do not have secure caps. A possible source of infection may also be the inappropriate decontamination of the outer walls of straws before loading and expelling.

4. Liquid nitrogen in storage tanks likely contains a number of commensal and potentially pathogenic environmental microorganisms [114].
5. Cases of liquid nitrogen-mediated transmission of pathogens [115–117] have been documented but never in relation to cryopreserved oocytes or embryos. Disease transfer has occurred only in one occasion, where two leaky bags containing blood samples were stored in the same dewar [115].
6. According to the experiments of Bielanski [118], cross-contamination may also occur during storage between open embryo storage if one of them is artificially infected. However, the volume of the microbes was artificially extremely high, a concentration that may never happen in a clinical situation.
7. Not a single case of any disease transmission in ART has been found to be related to liquid nitrogen-mediated cross-contamination, in spite of the enormous amount of human sperm samples, embryos, and oocytes stored worldwide, neither related to traditional, supposedly closed (but very often leaky or inappropriately handled) systems, nor with the open vitrification systems, in spite of the enormous focus on the latter. A study published in 2012 provided evidence from a real-case scenario of the lack of risk of cross-contamination among seropositive patients, even using an open device for vitrification [119].

There is no doubt that closed and properly handled systems should always be preferred, provided the outcome is comparable with the open systems. Results achieved by using closed systems for cleavage-stage human embryos and blastocysts are promising [108, 120, 121]. However, the fact that for human oocyte vitrification, open systems are superior than closed ones proves the superiority of the former [69, 122]. A possible solution is to make cooling in sterilized liquid nitrogen [123, 124] and store the samples in pre-cooled, hermetically sealed containers afterwards [83, 125]. Alternatively, open carriers can also be stored in cryo-tanks where instead of liquid, the vapour of nitrogen maintains the low storage temperature [126]. There have been a number of studies demonstrating the efficiency of the vapour storage system for vitrified oocytes/embryos using open carriers both in animal and human systems [126–128]. Concerns may also be raised regarding the applicability of closed systems for other chilling-sensitive objects including cattle oocytes and early-stage embryos, porcine blastocysts, or human oocytes [129], regarding not just *in vitro* survival rates but also *in vitro* development, pregnancies, and birth of healthy offspring.

Warming

Early on, Rall [57] found that high survival of vitrified embryos can be achieved with rather slow warming rates. However, most vitrification methods use rapid warming procedures, and recently it has been demonstrated that warming rates may be even more important than cooling rates [130].

Closed systems are usually immersed into water baths, while open systems can be directly submerged into the medium; this way the warming and the first dilution is performed in a single

step. The seemingly negligible difference may contribute considerably to the inferior results achieved with some closed systems consisting of a simple plastic or glass tube. After warming in the water bath, the surface has to be decontaminated quickly with a non-toxic but perfectly safe disinfectant, then the tube is cut and the sample is expelled into the appropriate medium. It means a significant delay between the warming and dilution; accordingly, the samples in this critical, very fragile phase are exposed for a relatively long period of time (5–10 seconds) to the concentrated cryo-protectants.

Although a slight devitrification (occurrence of ice crystals) may occur, especially when the cryo-protectant level is kept at the minimum level, this transitional change is usually restricted to a part of the embryo-containing medium and most probably does not involve intracellular crystal formation, and consequently does not cause significant harm in the embryos or oocytes [131], especially if the volume of the droplet is minimized (at the time of placing the embryo on the carrier).

In routine warming protocols of vitrified embryos, the dilution is a multistep procedure with decreasing concentration of osmotic buffers (usually sucrose) to counterbalance the swelling caused by the permeable cryo-protectant that leaves the cells relatively slowly. This delicate multistep dilution procedure seems to be indispensable for human embryos or oocytes, although one-step dilution without significant decrease of *in vitro* survival was reported in some animal species including cattle [48, 65, 132] and pigs [133]. Based on this approach, direct transfer methods after ultra-rapid vitrification of embryos resulted in offspring after transfer in cattle [134] and sheep [135]. Interestingly, the commonly used warming protocol (for vitrified samples) may also be used very efficiently for slow-frozen oocytes/embryos—thus providing a simplified and standard warming protocol for all samples, irrespective how they were frozen/vitrified [136].

Factors influencing the outcome

Species, genotype

There are well-demonstrated but poorly understood differences in sensitivity to cryo-injuries between different species in mammals. It appears that transparent oocytes and embryos are usually more resistant, and dense dark ones are more fragile, due to the increased lipid content. Accordingly, cryopreservation of light mouse embryos is a relatively easy task, cryopreservation of darker bovine embryo is a more difficult task, and the cryopreservation of dense pig embryos is truly a challenge in cryobiology. In parallel with the lighter appearance of the cytoplasm, considerably increased survival rates were detected after both slow freezing [137] and vitrification [138–141]. This approach also improves *in vitro* survival of vitrified porcine blastocysts produced by somatic cell nuclear transfer [141, 142].

It should be also noted that apart from the differences between species, in mice, differences between genotypes in the ability to develop after vitrification were also observed [143].

Developmental stage

The change in the size and shape of the cells is unprecedented in the first five to six days of mammalian development. A relatively simple spherical shape protected by an acellular outer layer develops to a complicated multicellular structure without external protection. Predictably, the extreme differences in morphology also correlate considerably with differences in sensitivity to cryo-injuries.

Generally, the earlier the development stage (starting from the germinal vesicle stage), the more sensitive oocytes and embryos are. However, although there is only a minimal difference between the size and shape, the immature oocytes are usually more sensitive to cryopreservation than mature (MII phase) oocytes [25, 129, 144]. Membrane permeability related to the type and expression levels of aquaporin at different stages may also explain differences in cryo-protectant protection efficiency and thus differences in survival [145, 146]. Additionally, a very remarkable difference exists between the chilling sensitivity of unfertilized and fertilized human oocytes. A possible explanation for this phenomenon is the increased chilling sensitivity of membranes: the lipid phase transition at room temperature storage in human germinal vesicle and MII stage oocytes is 10 times higher than that of human pronuclear embryos [129].

In the human, the survival rates after slow freezing are not significantly different between zygotes, cleavage-stage embryos, and blastocysts (between 65% and 85% for each; [147–149]). Similarly, in the human, survival rates after vitrification are not different for zygote-, cleavage-stage or blastocyst-stage embryos (though at each stage, vitrification provides a significantly higher survival rate; [150–152]). The complex structure of blastocysts may give rise to additional problems. In humans, mechanical reduction of the blastocoel by puncturing or repeated pipetting improved survival and pregnancy rates [134, 153–156]. The usual explanation is that the large blastocoel may not be protected appropriately from ice crystal formation [153]. Survival rates of blastocyst-stage embryos using vitrification are extremely high (it is usually above 95%), even without any additional “manipulation” [149]. However, some studies demonstrated that blastocyst survival (and intactness) may be further increased after vitrification, if the blastocoel is punctured, resulting in shrinkage—especially when blastocyst is expanded—in human and in other species as well [156–159].

In vivo- versus *in vitro*-produced embryos

Due to the lack of *in vivo*-derived human embryos, such differences can only be evaluated in domestic and experimental animals [160]. In these species, *in vivo*-produced embryos are more resistant to injuries—including cryo-injuries—than their *in vitro*-fertilized or cloned counterparts. Again, there might be some correlation between the increased lipid content of embryos produced in some *in vitro* systems. In general, the less morphological difference from the *in vivo* counterpart is detectable in the *in vivo*-produced embryos, the smaller the expected difference in survival after cryopreservation [161]. Although total elimination of these differences is still impossible, according to the joint conclusion of many publications, vitrification seems to be especially appropriate to counterbalance this handicap [162].

Outcomes after embryo vitrification

Domestic, experimental, and wild animals

There is an extensive literature of comparative experiments between slow freezing versus vitrification (some examples may include [91, 92, 143, 163–168]). The overwhelming majority of these papers prove the superiority of vitrification for the given purpose. Probably less than 10% of the studies did not find significant differences that were conducted at an early stage; however, the overwhelming majority of more recent studies clearly demonstrated the superiority of vitrification. Moreover, there are situations where vitrification is uniquely or predominantly suitable to achieve the goal: most of these areas are summarized in Table 22.2.

TABLE 22.2 Examples in Mammalian Embryology Where First Success in Cryopreservation Was Achieved by Vitrification.

Species, stage, system	Reference
Bovine immature oocytes for IVF	Vieria et al. [249]
Bovine <i>in vitro</i> matured oocytes for IVF	Martino et al. [250]; Vajta et al. [83]
Bovine <i>in vitro</i> matured oocytes for somatic cell nuclear transfer	Hou et al. [251]
Bovine cytoplasts for embryonic cell nuclear transfer	Booth et al. [252]
Bovine early-stage IVF embryos	Vajta et al. [84]; <i>in vitro</i> study
Bovine zona-included blastocysts generated by somatic cell nuclear transfer	French et al. [253]
Bovine zona-free blastocysts generated by somatic cell nuclear transfer	Tecirlioglu et al. [134]
Bovine transgenic blastocysts generated by somatic cell nuclear transfer	French et al. [254]
Ovine zona included embryos generated by nuclear transfer	Peura et al. [255]
Porcine immature oocytes for ICSI	Fujihira et al. [256]; <i>in vitro</i> study
Porcine <i>in vitro</i> matured oocytes for ICSI	Fujihira et al. [257]; <i>in vitro</i> study
Porcine <i>in vivo</i> -derived blastocysts	Kobayashi et al. [258]
Porcine <i>in vivo</i> -derived morulae	Berthelot et al. [259]
Porcine <i>in vitro</i> produced blastocysts	Men et al. [260]; <i>in vitro</i> study
Equine <i>in vivo</i> -matured oocytes	Maclellan et al. [261]
European polecat <i>in vivo</i> -derived morulae and blastocysts	Piltty et al. [262]
Siberian tiger <i>in vivo</i> -derived embryos	Crichton et al. [263]; <i>in vitro</i> study
Minke whale immature oocytes for maturation	Iwayama et al. [264]; <i>in vitro</i> study

Abbreviation: ICSI = intracytoplasmic sperm injection.

Source: Reprinted from Vajta and Nagy [248] with permission from Reproductive Healthcare Ltd.

Note: Embryos and Oocytes Were Not Treated Mechanically or Chemically to Prepare Them for the Vitrification. Full-Term Developments Were Reported Except Where Otherwise Indicated

Human embryos

In humans, the clinical pregnancy rate from embryo transfer after slow-freezing has been approximately two-thirds that from the fresh transfer of embryos [169], although some techniques have helped to restore (cleavage stage) embryo viability [170, 171]. The theoretical possibility for improvement is supported by the results obtained in cattle, where the difference is no more than 10%–15%.

However, vitrification has a clear superiority for embryo cryopreservation and for this reason, it is the standard in most parts of the world [5, 22, 23, 79, 80, 88, 89, 93, 94, 100, 134, 153, 165, 172–184].

Early on, in 2005, three comparative investigations were published, and all three concluded that vitrification was a more efficient way for cryopreservation of human embryos than slow-rate

freezing [69, 185, 186]. More recent comparative studies published in the literature have confirmed that vitrification is clearly more efficient than slow freezing used at different embryonic development stages [187–192]. Accordingly, these representative comparisons have proved that vitrification is more efficient than slow-rate freezing for the cryopreservation of human embryos at all stages [193]. In addition to those comparative studies, other, non-comparative studies on the efficiency of vitrification have been published, applying the technique at different stages of pre-implantation embryo development, including zygote, cleavage, and blastocyst stages [121, 151, 155, 192, 194–198], including also day-7 successful vitrification [199]. Based on these reported improved outcomes, a consensus meeting was organized by Alpha Scientist, to set minimum standards and inspirational outcome parameters (KPIs) following cryopreservation, that today set the standards worldwide [149].

Although several tools (carriers) and kits (vitrification/warming solutions) are currently available for vitrification, two technologies related to the type of carriers have obtained more attention initially: the OPS, predominantly in the animal field, and the Cryotop (and similarly formed other cryo-devices) for human areas. The more delicate Cryotop method may be the preferred choice where extremely high cooling rates are the primary objectives. As written earlier, there are now several new cryo-tools/carriers available on the market, which have been tested and used more widely for both embryo and oocyte vitrification. Storage time, as expected, had no impact on outcomes of vitrified embryos (or vitrified oocytes), as different studies have established [200, 201], if samples are stored and handled adequately, avoiding accidental warming.

Reproductive Biology Associates (RBA), an IVF Clinic located in Georgia, was one of the very first in the United States (and in the world) to apply embryo (and oocyte) vitrification in routine patient care. Initial data, when employing both techniques (slow freezing and vitrification) on the same time period (2006–2007) demonstrates significantly better outcomes with vitrification compared to slow freezing in comparable patient population (Table 22.3).

Vitrification and ART services

Routine application of vitrification has spread out all over the world in recent years [192, 202, 203], resulting in a paradigm shift in how assisted reproduction treatment is performed. The extreme high efficiency of vitrification applied on oocytes and embryos provides the possibilities for novel patient services. Oocyte and embryo vitrification now can provide the base for fertility preservation for both medical and social reasons [204–208] and for donor egg banking [101, 103], or for various other clinical conditions, such as hyperstimulation, failure to obtain sperm on the day of oocyte collection, or due to moral/ethical reasons for preferring egg preservation instead of embryo preservation [209–212], cryopreserving excess oocytes aspirated from IUI patients with excess follicles [213].

The highly efficient embryo vitrification, has opened up several new possibilities. One of the most important benefits relates to embryo biopsy and PGS/PGD. In the past, survival of embryos after slow-freeze/thaw following embryo biopsy was more than disappointing, strongly limiting the use of biopsy and genetic testing—mainly to be performed on day-3 cleavage stage, or for polar body biopsy [214, 215]. Applying vitrification instead of slow-freezing on biopsied embryos has significantly improved survival rates [185, 216], thus this procedure has become

TABLE 22.3 Outcomes of Embryo Cryopreservation—289 Cycles of Slow Freezing and 108 Cycles of Vitrification (1494 Frozen/Thawed and 418 Vitrified/Warmed Embryos)

Embryo Stage	Outcomes	Vitrification	Slow Freezing	P-Value
2PN	Survival rate	97%	85%	<0.001
	Clinical pregnancy rate	59%	43%	0.1864
	Implantation rate per embryo transferred	28%	23%	0.3921
	Implantation rate per embryo thawed	14%	10%	0.171
Cleavage	Survival rate	90%	75%	0.0044
	Clinical pregnancy rate	50%	43%	0.422
	Implantation rate per embryo transferred	32%	22%	0.0889
	Implantation rate per embryo thawed	26%	12%	0.0021
Blastocyst	Survival rate	93%	76%	<0.001
	Clinical pregnancy rate	65%	55%	0.2802
	Implantation rate per embryo transferred	36%	33%	0.6963
	Implantation rate per embryo thawed	33%	26%	0.1743
All Stages	Survival rate	94%	80%	<0.001
	Clinical pregnancy rate	61%	45%	0.0025
	Implantation rate per embryo transferred	33%	24%	0.0032
	Implantation rate per embryo thawed	23%	12%	<0.001

routine when embryos are to be tested genetically [190, 217]. Additionally, now biopsy timing can be shifted from day 3 (or day 0/day 1) to day 5/day 6, when embryos develop to the blastocyst stage, as there is no more need to use these embryos for fresh transfer, as they will survive cryopreservation much better, usually perfectly. Blastocyst stage embryo biopsy has several advantages compared to earlier stages, specially to day-3 stage biopsy, as embryos are more resistant to the biopsy procedure, more cells can be removed (genetic testing can be more reliable); less likely to encounter mosaicism; and embryos will be transferred in a (possibly) more receptive uterine environment. All these factors in combination result in very high pregnancy rates [217–220]. Pregnancies and live births were reported also when vitrification was repeated on the same embryo at the same (or different) stage or after oocyte vitrification, or even after

involving a trophectoderm biopsy, demonstrating the robustness of the technique [104, 220–223]. Because of the extremely high success rates obtained with vitrified embryos after biopsy, it seems a logical extension of the thinking, that other patients with different clinical conditions may also benefit of the “cryo-preserve all” embryos and perform transfer in a “cryo-cycle” [224]. Rationally, patients at risk for ovarian hyperstimulation can clearly benefit from vitrifying all embryos [198]. Other studies suggest “cryo-embryos” for women with endometriosis [225], while some may consider to apply this idea for all IVF patients, looking for the benefit of a more receptive endometrium in a cryo-cycle for patients who underwent ovarian stimulation [224, 226]. Additionally, patients with diminished ovarian reserve (irrespective of reproductive age) may also benefit, for the same reasons, from the “cryo-all” strategy. Instead of performing a fresh transfer, all embryos or oocytes (possibly vitrified at an early stage, due to the low oocyte/zygote number) are cryopreserved and then transferred later in a “frozen embryo transfer cycle”/“FER cycle” (either natural or supplemented cycle; [227, 228]). In fact, there are less and less reasons to perform fresh embryo transfer; this is the primary reason that today in most countries the “cryo-all” strategy is applied for most IVF patients—and, consequently, embryo transfers are performed in “FER cycles” [224]. In fact, when comparing live birth rates after fresh and “frozen” embryo transfer, data clearly demonstrates an overall superior outcome after “frozen” embryo transfer. For instance, in the United States, in 2001, the live birth rate following fresh transfer was 33.4%, and after “frozen” embryo transfer it was 23.4% (Figure 22.4). Fifteen years later, in 2016, the live birth rate after a fresh transfer was just very slightly increased to 36.3%; however, the “frozen” embryo transfer rate jumped to 45.9% (Figure 22.4 shows the “power” of vitrification). Moreover, the high efficiency of embryo vitrification also strongly promotes single embryo transfer. For instance, in the United States (where single embryo transfer is optional), the proportion of single embryo transfers increased from 18% (in 2010) to 77% (in 2019), as a direct consequence of having vitrification technology available (Figure 22.5.) In the past, when using slow-freezing, embryo survival was suboptimal, “promoting” higher numbers of embryos to be transferred as fresh, as it was not known how many of the cryopreserved supernumerary embryos would survive upon thawing (the other reason was the suboptimal embryo culture/development). Today, using vitrification, the viability of the embryo post-warming is virtually equivalent to the viability prior to cryopreservation, which in combination with a more “natural” endometrium can benefit patients and offspring [229]. The fact that in a 13-year period the number of “freeze all” IVF cycles increased 60× (from 2000 cycles in 2007 to 121,000 in 2019) is the direct consequence of the impact of vitrification IVF patient care, supported by other changes, such as improved embryo culture and the trend to cryopreserve embryos at the most potent stage, when developed to blastocyst; increasing use of pre-implantation genetic screening to deselect chromosomally abnormal embryos; and the effectiveness of vitrification, as well as taking advantage of a more receptive endometrium.

Safety of vitrification

For a new technique or technology to be fully accepted and applied worldwide, there are two critical points which need to be fulfilled: efficiency and safety. Vitrification of embryos/oocytes has now been clearly demonstrated to provide extremely high

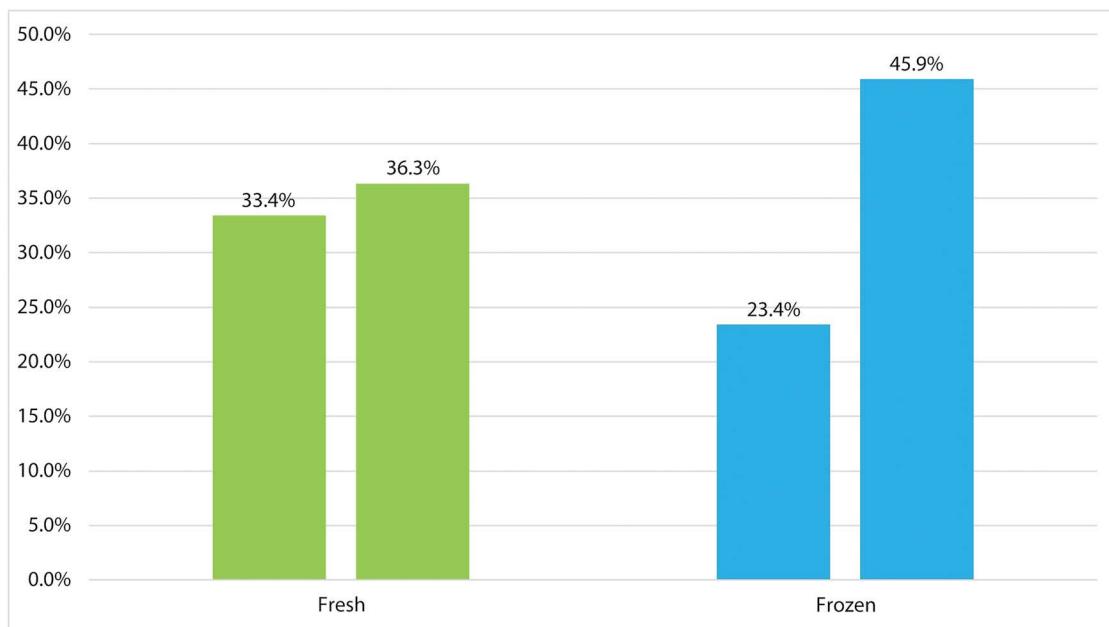


FIGURE 22.4 Live birth rates after fresh and “frozen” embryo transfers in the United States, 2001 and 2016.

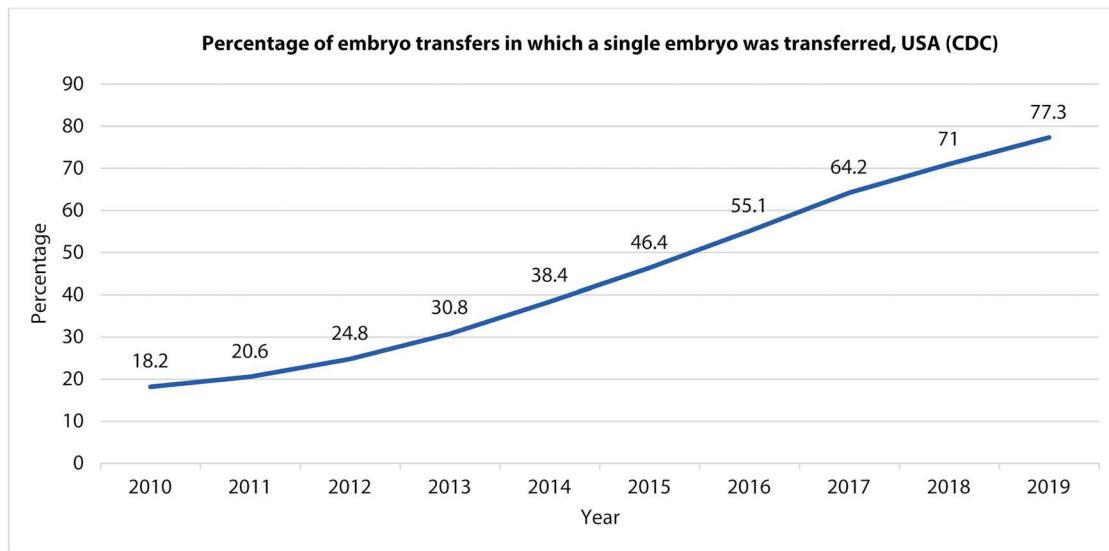


FIGURE 22.5 Proportion of embryo transfers with single embryo transferred in the United States, from 2010 to 2019.

efficiency, demonstrating outcomes similar to those achieved by using fresh oocytes/embryos. However, safety is a point yet to be proven beyond any doubt. Initial studies presenting outcomes on live birth data (mainly gathered after oocyte vitrification) do not indicate any alarming or unexpected results or trends [194, 220, 222, 230–234]. More recent data on babies born after embryo vitrification continue to demonstrate the safety of the technique, not showing increased risks for birth defects or other live birth parameters [235–238]. It would be most prudent for national or international IVF societies to organize the needed data collection through registry(ies). Only a multicentre effort where most IVF clinics participate would be able to provide a sufficient amount of data in a reasonable period of time.

Conclusions

Vitrification as an approach to cryopreserve human embryos or oocytes has achieved remarkable success. Today, vitrification is the “gold standard” in human-assisted reproduction, and most likely all (or virtually all) of IVF clinics are now using vitrification for reproductive tissue cryopreservation. This extraordinary achievement would not have been possible without the constant dedication and hard work of the few early pioneers, mainly coming from the field of veterinary medicine. On safety of vitrification, any currently available data do not indicate a higher incidence of malformation—which is reassuring, but obviously needs to be confirmed on a much larger scale.

The overwhelming majority of the studies/publications support the application of vitrification by emphasizing its advantages: the simple, inexpensive, and rapid procedure leading to higher survival and developmental rates than those achievable with alternative methods. Concerns regarding disease transmission are theoretically justified, but safer methods are now available to mitigate this risk. Outstanding results like the breakthrough in human oocyte vitrification and the excellent (and improved) results on embryo cryopreservation have changed the way how we practice routine IVF, providing more efficient and safer options for the patients.

References

- Roy TK, et al. Embryo vitrification using a novel semi-automated closed system yields in vitro outcomes equivalent to the manual cryotop method. *Hum Reprod*. 2014;29(11):2431–8.
- Gatimel N, et al. Semi-automated versus manual embryo vitrification: Inter-operator variability, time-saving, and clinical outcomes. *J Assist Reprod Genet*. 2021;38(12):3213–22.
- Shaw JM, Jones GM. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. *Hum Reprod Update*. 2003;9(6):583–605.
- Fuller B, Paynter S. Fundamentals of cryobiology in reproductive medicine. *Reprod Biomed Online*. 2004;9(6):680–91.
- Kasai M, Mukaida T. Cryopreservation of animal and human embryos by vitrification. *Reprod Biomed Online*. 2004;9(2):164–70.
- Smith GD, Silva ESCA. Developmental consequences of cryopreservation of mammalian oocytes and embryos. *Reprod Biomed Online*. 2004;9(2):171–8.
- Stachecki JJ, Cohen J. An overview of oocyte cryopreservation. *Reprod Biomed Online*. 2004;9(2):152–63.
- Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos frozen to -196 degrees and -269 degrees C. *Science*. 1972;178(59):411–4.
- Wilmut I. The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. *Life Sci II*. 1972;11(22):1071–9.
- Wilmut I, Rowson LE. Experiments on the low-temperature preservation of cow embryos. *Vet Rec*. 1973;92(26):686–90.
- Bank H, Maurer RR. Survival of frozen rabbit embryos. *Experimental Cell Research*. 1974;89(1):188–96.
- Willadsen SM, et al. Deep freezing of sheep embryos. *J Reprod Fertil*. 1976;46(1): 151–4.
- Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature*. 1983;305(5936):707–9.
- Zeilmaker GH, et al. Two pregnancies following transfer of intact frozen-thawed embryos. *Fertil Steril*. 1984;42(2):293–6.
- Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 degrees c by vitrification. *Nature*. 1985;313(6003):573–5.
- Mazur P. Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. *Cell Biophys*. 1990;17(1):53–92.
- Leibo SP, McGrath JJ, Cravalho EG. Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. *Cryobiology*. 1978;15(3):257–71.
- Kasai M, Niwa K, Iritani A. Survival of mouse embryos frozen and thawed rapidly. *J Reprod Fertil*. 1980;59(1):51–6.
- Wood MJ, Farrant J. Preservation of mouse embryos by two-step freezing. *Cryobiology*. 1980;17(2):178–80.
- Trounson A, Peura A, Kirby C. Ultrarapid freezing: A new low-cost and effective method of embryo cryopreservation. *Fertil Steril*. 1987;48(5):843–50.
- Shaw JM, Diotallevi L, Trounson A. Ultrarapid embryo freezing: Effect of dissolved gas and pH of the freezing solutions and straw irradiation. *Hum Reprod*. 1988;3(7):905–8.
- Barg PE, Barad DH, Feichtinger W. Ultrarapid freezing (URF) of mouse and human preembryos: A modified approach. *J in vitro Fertil Embryo Transf*. 1990;7(6):355–7.
- Feichtinger W, Hochfellner C, Ferstl U. Clinical experience with ultra-rapid freezing of embryos. *Hum Reprod*. 1991;6(5):735–6.
- Aman RR, Parks JE. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. *Biol Reprod*. 1994;50(1):103–10.
- Martino A, Pollard JW, Leibo SP. Effect of chilling bovine oocytes on their developmental competence. *Mol Reprod Dev*. 1996;45(4):503–12.
- Zenze MT, et al. Effects of chilling to 0 degrees c on the morphology of meiotic spindles in human metaphase II oocytes. *Fertil Steril*. 2001;75(4):769–77.
- Rall WF, Meyer TK. Zona fracture damage and its avoidance during the cryopreservation of mammalian embryos. *Theriogenology*. 1989;31(3):683–92.
- Sansinena M, et al. Implications of storage and handling conditions on glass transition and potential devitrification of oocytes and embryos. *Theriogenology*. 2014;82(3):373–8.
- Rall, W.F. Cryopreservation of mammalian embryos, gametes and ovarian tissues. Current issues and progress. In: Assisted Fertilization and Nuclear Transfer in Mammals. Wolf DP, Zelinski-Wooten M. (eds.). Humana Press: Totowa, NJ, pp. 173–187, 2001.
- Chaytor JL, et al. Inhibiting ice recrystallization and optimization of cell viability after cryopreservation. *Glycobiology*. 2012;22(1):123–33.
- Cha SK, et al. Effects of various combinations of cryoprotectants and cooling speed on the survival and further development of mouse oocytes after vitrification. *Clin Exp Reprod Med*. 2011;38(1):24–30.
- Vincent C, Johnson MH. Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. *Oxf Rev Reprod Biol*. 1992;14:73–100.
- Massip A, Mermilliod P, Dinnyes A. Morphology and biochemistry of in-vitro produced bovine embryos: Implications for their cryopreservation. *Hum Reprod*. 1995;10(11):3004–11.
- Chang CC, et al. The oocyte spindle is preserved by 1,2-propanediol during slow freezing. *Fertil Steril*. 2010;93(5):1430–9.
- Gardner DK, et al. Analysis of oocyte physiology to improve cryopreservation procedures. *Theriogenology*. 2007;67(1):64–72.
- Truong T, Harvey AJ, Gardner DK. Antioxidant supplementation of mouse embryo culture or vitrification media support more in-vivo-like gene expression post-transfer. *Reprod Biomed Online*. 2022;44(3):393–410.
- dela Pena EC, et al. Vitrification of mouse oocytes in ethylene glycol-raffinose solution: Effects of preexposure to ethylene glycol or raffinose on oocyte viability. *Cryobiology*. 2001;42(2):103–11.
- Ishimori H, Takahashi Y, Kanagawa H. Factors affecting survival of mouse blastocysts vitrified by a mixture of ethylene glycol and dimethyl sulfoxide. *Theriogenology*. 1992;38(6):1175–85.
- Ishimori H, et al. Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxide. *Theriogenology*. 1993;40(2):427–33.
- Vicente JS, Garcia-Ximenez F. Osmotic and cryoprotective effects of a mixture of DMSO and ethylene glycol on rabbit morulae. *Theriogenology*. 1994;42(7):1205–15.
- Aye M, et al. Assessment of the genotoxicity of three cryoprotectants used for human oocyte vitrification: Dimethyl sulfoxide, ethylene glycol and propylene glycol. *Food Chem Toxicol*. 2010;48(7):1905–12.
- Lawson A, Ahmad H, Sambanis A. Cytotoxicity effects of cryoprotectants as single-component and cocktail vitrification solutions. *Cryobiology*. 2011;62(2):115–22.
- Ali J, Shelton JN. Design of vitrification solutions for the cryopreservation of embryos. *J Reprod Fertil*. 1993;99(2):471–7.
- Kasai M. Cryopreservation of mammalian embryos. *Mol Biotechnol*. 1997;7(2):173–9.

45. Wright DL, et al. Use of sugars in cryopreserving human oocytes. *Reprod Biomed Online.* 2004;9(2):179–86.
46. Kasai M. Nonfreezing technique for short-term storage of mouse embryos. *J in vitro Fertil Embryo Transf.* 1986;3(1):10–4.
47. Kasai M, et al. Survival of mouse morulae vitrified in an ethylene glycol-based solution after exposure to the solution at various temperatures. *Biol Reprod.* 1992;47(6):1134–9.
48. Vajta G, et al. Survival and development of bovine blastocysts produced in vitro after assisted hatching, vitrification and in-straw direct rehydration. *J Reprod Fertil.* 1997;111(1):65–70.
49. Kuleshova LL, et al. Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. *Cryobiology.* 1999;38(2):119–30.
50. Oda K, Gibbons WE, Leibo SP. Osmotic shock of fertilized mouse ova. *J Reprod Fertil.* 1992;95(3):737–47.
51. Ohboshi S, et al. Usefulness of polyethylene glycol for cryopreservation by vitrification of in vitro-derived bovine blastocysts. *Anim Reprod Sci.* 1997;48(1):27–36.
52. Shaw JM, et al. Vitrification properties of solutions of ethylene glycol in saline containing PVP, ficoll, or dextran. *Cryobiology.* 1997;35(3):219–29.
53. Naitana S, et al. Polyvinyl alcohol as a defined substitute for serum in vitrification and warming solutions to cryopreserve ovine embryos at different stages of development. *Anim Reprod Sci.* 1997;48(2-4):247–56.
54. Kuleshova LL, Shaw JM, Trounson AO. Studies on replacing most of the penetrating cryoprotectant by polymers for embryo cryopreservation. *Cryobiology.* 2001;43(1):21–31.
55. Asada M, et al. Effect of polyvinyl alcohol (PVA) concentration during vitrification of in vitro matured bovine oocytes. *Theriogenology.* 2002;58(6):1199–208.
56. Kasai M, et al. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J Reprod Fertil.* 1990;89(1):91–7.
57. Rall WF. Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology.* 1987;24(5):387–402.
58. Lane M, et al. Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. *Mol Reprod Dev.* 2003;64(1):70–8.
59. Rubinsky B, Arav A, Devries AL. The cryoprotective effect of antifreeze glycopeptides from Antarctic fishes. *Cryobiology.* 1992;29(1):69–79.
60. Eto TK, Rubinsky B. Antifreeze glycoproteins increase solution viscosity. *Biochem Biophys Res Commun.* 1993;197(2):927–31.
61. Wowk B, et al. Vitrification enhancement by synthetic ice blocking agents. *Cryobiology.* 2000;40(3):228–36.
62. Mori C, et al. Hydroxypropyl cellulose as an option for supplementation of cryoprotectant solutions for embryo vitrification in human assisted reproductive technologies. *Reprod Biomed Online.* 2015;30(6):613–21.
63. Coello A, et al. A combination of hydroxypropyl cellulose and trehalose as supplementation for vitrification of human oocytes: A retrospective cohort study. *J Assist Reprod Genet.* 2016;33(3):413–21.
64. Vanderzwalmen P, et al. Vitrification of bovine blastocysts. *Theriogenology.* 1998;31:270.
65. Saha S, et al. Direct rehydration of in vitro fertilised bovine embryos after vitrification. *Vet Rec.* 1994;134(11):276–7.
66. Szell AZ, Windsor DP. Survival of vitrified sheep embryos in vitro and in vivo. *Theriogenology.* 1994;42(5):881–9.
67. Papis K, Shimizu M, Izaike Y. Factors affecting the survivability of bovine oocytes vitrified in droplets. *Theriogenology.* 2000;54(5):651–8.
68. Kuwayama M, et al. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online.* 2005;11(3):300–8.
69. Kuwayama M, et al. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reprod Biomed Online.* 2005;11(5):608–14.
70. Vanderzwalmen P, et al. Lower intracellular concentration of cryoprotectants after vitrification than after slow freezing despite exposure to higher concentration of cryoprotectant solutions. *Hum Reprod.* 2013;28(8):2101–10.
71. Miao S, et al. A Robotic System With Embedded Open Microfluidic Chip for Automatic Embryo Vitrification. *IEEE Trans Biomed Eng.* 2022;69(12):3562–71.
72. Palasz AT, Mapletoft RJ. Cryopreservation of mammalian embryos and oocytes: Recent advances. *Biotechnol Adv.* 1996;14(2):127–49.
73. Cuello C, et al. Vitrification of porcine embryos at various developmental stages using different ultra-rapid cooling procedures. *Theriogenology.* 2004;62(1-2):353–61.
74. Liu WX, et al. Effects of different cryoprotectants and cryopreservation protocols on the development of 2-4 cell mouse embryos. *Cryo Letters.* 2011;32(3):240–7.
75. Arav A, et al. A new, simple, automatic vitrification device: Preliminary results with murine and bovine oocytes and embryos. *J Assist Reprod Genet.* 2018;35(7):1161–8.
76. Landa V, Teplo O. Cryopreservation of mouse 8-cell embryos in microdrops. *Folia Biol (Praha).* 1990;36(3-4):153–8.
77. Riha J. Vitrification of cattle embryos by direct dropping into liquid nitrogen and embryo survival after nonsurgical transfer. *Zivot Viroba.* 1994;36:113–20.
78. Yang BC, Leibo SP. Viability of in vitro-derived bovine zygotes cryopreserved in microdrops. *Theriogenology.* 1999;51:178.
79. Choi DH, et al. Pregnancy and delivery of healthy infants developed from vitrified blastocysts in an IVF-ET program. *Fertil Steril.* 2000;74(4):838–9.
80. Cho HJ, et al. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. *Hum Reprod.* 2002;17(9):2419–22.
81. Son WY, et al. Pregnancy resulting from transfer of repeat vitrified blastocysts produced by in-vitro matured oocytes in patient with polycystic ovary syndrome. *Reprod Biomed Online.* 2005;10(3):398–401.
82. Vajta G, et al. Vitrification of porcine embryos using the open pulled straw (OPS) method. *Acta Vet Scand.* 1997;38(4):349–52.
83. Vajta G, et al. Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev.* 1998;51(1):53–8.
84. Vajta G, et al. Sterile application of the open pulled straw (OPS) vitrification method. *Cryo Letters.* 1998;19:389–92.
85. Arav A, Zeron Y, Ocheretny A. A new device and method for vitrification increases the cooling rate and allows successful cryopreservation of bovine oocytes. *Theriogenology.* 2000;53:248.
86. Arav A, et al. New trends in gamete's cryopreservation. *Mol Cell Endocrinol.* 2002;187(1-2):77–81.
87. Chen SU, et al. Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. *Hum Reprod.* 2000;15(12):2598–603.
88. El-Danasouri I, Selman H. Successful pregnancies and deliveries after a simple vitrification protocol for day 3 human embryos. *Fertil Steril.* 2001;76(2):400–2.
89. Selman HA, El-Danasouri I. Pregnancies derived from vitrified human zygotes. *Fertil Steril.* 2002;77(2):422–3.
90. Isachenko V, et al. Modified vitrification of human pronuclear oocytes: Efficacy and effect on ultrastructure. *Reprod Biomed Online.* 2003;7(2):211–6.
91. Lane M, et al. Containerless vitrification of mammalian oocytes and embryos. *Nat Biotechnol.* 1999;17(12):1234–6.
92. Lane M, Schoolcraft WB, Gardner DK. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril.* 1999;72(6):1073–8.
93. Mukaida T, et al. Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertil Steril.* 2001;76(3):618–20.

94. Mukaida T, Takahashi K, Kasai M. Blastocyst cryopreservation: Ultrarapid vitrification using cryoloop technique. *Reprod Biomed Online.* 2003;6(2):221–5.
95. Isachenko E, et al. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: From past practical difficulties to present success. *Reprod Biomed Online.* 2003;6(2):191–200.
96. Larman MG, Sheehan CB, Gardner DK. Vitrification of mouse pronuclear oocytes with no direct liquid nitrogen contact. *Reprod Biomed Online.* 2006;12(1):66–9.
97. Matsumoto H, et al. Vitrification of large quantities of immature bovine oocytes using nylon mesh. *Cryobiology.* 2001;42(2):139–44.
98. Arav A, Shehu D, Mattioli M. Osmotic and cytotoxic study of vitrification of immature bovine oocytes. *J Reprod Fertil.* 1993;99(2):353–8.
99. Hamawaki A, Hamano KM. Minimum volume cooling method for bovine blastocyst vitrification. *Theriogenology.* 1999;51:165.
100. Vanderzwalmen P, et al. In vitro survival of metaphase II oocytes (MII) and blastocysts after vitrification in an hemi-straw (HS) system. *Fertil Steril.* 2000;74:S215–216.
101. Nagy ZP, et al. Clinical evaluation of the efficiency of an oocyte donation program using egg cryo-banking. *Fertil Steril.* 2009;92(2):520–6.
102. Chang CC, et al. Human oocyte vitrification: In-vivo and In-vitro maturation outcomes. *Reprod Biomed Online.* 2008;17(5):684–8.
103. Cobo A, et al. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the cryotop method. *Fertil Steril.* 2008;89(6):1657–64.
104. Chang CC, et al. Two successful pregnancies obtained following oocyte vitrification and embryo re-vitrification. *Reprod Biomed Online.* 2008;16(3):346–9.
105. Gutnisky C, et al. Evaluation of the cryotech vitrification kit for bovine embryos. *Cryobiology.* 2013;67(3):391–3.
106. Larman MG, Gardner DK. Vitrification of mouse embryos with super-cooled air. *Fertil Steril.* 2011;95(4):1462–6.
107. Hashimoto S, et al. A closed system supports the developmental competence of human embryos after vitrification: Closed vitrification of human embryos. *J Assist Reprod Genet.* 2013;30(3):371–6.
108. Van Landuyt L, et al. Outcome of closed blastocyst vitrification in relation to blastocyst quality: Evaluation of 759 warming cycles in a single-embryo transfer policy. *Hum Reprod.* 2011;26(3):527–34.
109. Schiwe MC, et al. Validation of microSecure vitrification (muS-VTF) for the effective cryopreservation of human embryos and oocytes. *Cryobiology.* 2015;71(2):264–72.
110. Bonetti A, et al. Ultrastructural evaluation of human metaphase II oocytes after vitrification: Closed versus open devices. *Fertil Steril.* 2011;95(3):928–35.
111. Papatheodorou A, et al. Open versus closed oocyte vitrification system: A prospective randomized sibling-oocyte study. *Reprod Biomed Online.* 2013;26(6):595–602.
112. Huang CC, et al. Successful pregnancy following blastocyst cryopreservation using super-cooling ultra-rapid vitrification. *Hum Reprod.* 2005;20(1):122–8.
113. Dinnyes A, et al. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. *Biol Reprod.* 2000;63(2):513–8.
114. Bielanski A, et al. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology.* 2003;46(2):146–52.
115. Tedder RS, et al. Hepatitis B transmission from contaminated cryopreservation tank. *Lancet.* 1995;346(8968):137–40.
116. Fountain D, et al. Liquid nitrogen freezers: A potential source of microbial contamination of hematopoietic stem cell components. *Transfusion.* 1997;37(6):585–91.
117. Berry ED, et al. Bacterial cross-contamination of meat during liquid nitrogen immersion freezing. *J Food Prot.* 1998;61(9):1103–8.
118. Bielanski A, et al. Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology.* 2000;40(2):110–6.
119. Cobo A, et al. Viral screening of spent culture media and liquid nitrogen samples of oocytes and embryos from hepatitis B, hepatitis C, and human immunodeficiency virus chronically infected women undergoing in vitro fertilization cycles. *Fertil Steril.* 2012;97(1):74–8.
120. Liebermann J. Vitrification of human blastocysts: An update. *Reprod Biomed Online.* 2009;19(Suppl 4):4328.
121. Vanderzwalmen P, et al. Aseptic vitrification of blastocysts from infertile patients, egg donors and after IVM. *Reprod Biomed Online.* 2009;19(5):700–7.
122. Vajta G, Rienzi L, Ubaldi FM. Open versus closed systems for vitrification of human oocytes and embryos. *Reprod Biomed Online.* 2015;30(4):325–33.
123. Parmegiani L, et al. Sterilization of liquid nitrogen with ultraviolet irradiation for safe vitrification of human oocytes or embryos. *Fertil Steril.* 2010;94(4):1525–8.
124. Parmegiani L, et al. Efficiency of aseptic open vitrification and hermetical cryostorage of human oocytes. *Reprod Biomed Online.* 2011;23(4):505–12.
125. Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. *Fertil Steril.* 2002;78(3):449–54.
126. Cobo A, et al. Storage of human oocytes in the vapor phase of nitrogen. *Fertil Steril.* 2010;94(5):1903–7.
127. Eum JH, et al. Long-term liquid nitrogen vapor storage of mouse embryos cryopreserved using vitrification or slow cooling. *Fertil Steril.* 2009;91(5):1928–32.
128. Abdelhafez F, et al. Vitrification in open and closed carriers at different cell stages: Assessment of embryo survival, development, DNA integrity and stability during vapor phase storage for transport. *BMC Biotechnol.* 2011;11:29.
129. Ghettler Y, et al. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Hum Reprod.* 2005;20(12):3385–9.
130. Mazur P, Seki S. Survival of mouse oocytes after being cooled in a vitrification solution to -196 degrees C at 95 degrees to 70,000 degrees C/min and warmed at 610 degrees to 118,000 degrees C/min: A new paradigm for cryopreservation by vitrification. *Cryobiology.* 2011;62(1):1–7.
131. Shaw JM, et al. An association between chromosomal abnormalities in rapidly frozen 2-cell mouse embryos and the ice-forming properties of the cryoprotective solution. *J Reprod Fertil.* 1991;91(1):9–18.
132. Vajta G, et al. In-straw dilution of bovine blastocysts after vitrification with the open-pulled straw method. *Vet Rec.* 1999;144(7):180–1.
133. Cuello C, et al. In vitro development following one-step dilution of OPS-vitrified porcine blastocysts. *Theriogenology.* 2004;62(6):1144–52.
134. Tecirlioglu RT, et al. Birth of a cloned calf derived from a vitrified hand-made cloned embryo. *Reprod Fertil Dev.* 2003;15(7–8):361–6.
135. Isachenko V, et al. New technology for vitrification and field (microscope-free) warming and transfer of small ruminant embryos. *Theriogenology.* 2003;59(5–6):1209–18.
136. Parmegiani L, et al. Rapid warming increases survival of slow-frozen sibling oocytes: A step towards A single warming procedure irrespective of the freezing protocol? *Reprod Biomed Online.* 2014;28(5):614–23.
137. Nagashima H, et al. Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. *Biol Reprod.* 1994;51(4):618–22.
138. Dobrinsky JR, et al. Cryopreservation of swine embryos with reduced lipid content. *Theriogenology.* 1999;51:164.
139. Beeb LF, et al. Piglets born from centrifuged and vitrified early and peri-hatching blastocysts. *Theriogenology.* 2002;57(9):2155–65.
140. Esaki R, et al. Cryopreservation of porcine embryos derived from in vitro-matured oocytes. *Biol Reprod.* 2004;71(2):432–7.
141. Du Y, Zhang KP, et al. Successful vitrification of parthenogenetic porcine blastocysts produced from delipidated in vitro matured oocytes. *Reprod Fertil Dev.* 2006;18:153.

142. Li R, et al. Cloned transgenic swine via in vitro production and cryopreservation. *Biol Reprod.* 2006;75(2):226–30.
143. Dinnyes A, Wallace GA, Rall WF. Effect of genotype on the efficiency of mouse embryo cryopreservation by vitrification or slow freezing methods. *Mol Reprod Dev.* 1995;40(4):429–35.
144. Men H, Monson RL, Rutledge JJ. Effect of meiotic stages and maturation protocols on bovine oocyte's resistance to cryopreservation. *Theriogenology.* 2002;57(3):1095–103.
145. Edashige K, et al. Artificial expression of aquaporin-3 improves the survival of mouse oocytes after cryopreservation. *Biol Reprod.* 2003;68(1):87–94.
146. Edashige K, Sakamoto M, Kasai M. Expression of mRNAs of the aquaporin family in mouse oocytes and embryos. *Cryobiology.* 2000;40(2):171–5.
147. Veek LL. Does the developmental stage at freeze impact on clinical results post-thaw? *Reprod Biomed Online.* 2003;6(3):367–74.
148. Pool TB, Leibo SP. Cryopreservation and assisted human conception. Introduction. *Reproductive Biomedicine Online.* 2004;9(2):132–3.
149. Alpha Scientists in Reproductive Medicine. The alpha consensus meeting on cryopreservation key performance indicators and benchmarks: Proceedings of an expert meeting. *Reprod Biomed Online.* 2012;25(2):146–67.
150. Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Hum Reprod Update.* 2012;18(5):536–54.
151. Wang XL, et al. Outcomes of day 3 embryo transfer with vitrification using cryoleaf: A 3-year follow-up study. *J Assist Reprod Genet.* 2012;29(9):883–9.
152. Fernandez-Shaw S, et al. Ongoing and cumulative pregnancy rate after cleavage-stage versus blastocyst-stage embryo transfer using vitrification for cryopreservation: Impact of age on the results. *J Assist Reprod Genet.* 2015;32(2):177–84.
153. Vanderzwalmen P, et al. Births after vitrification at morula and blastocyst stages: Effect of artificial reduction of the blastocoelic cavity Before vitrification. *Hum Reprod.* 2002;17(3):744–51.
154. Hiraoka K, Kinutani M, Kinutani K. Blastocoele collapse by micro-pipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Hum Reprod.* 2004;19(12):2884–8.
155. Raju GA, et al. Vitrification of human early cavitating and deflated expanded blastocysts: Clinical outcome of 474 cycles. *J Assist Reprod Genet.* 2009;26(9–10):523–9.
156. Iwayama H, Hochi S, Yamashita M. In vitro and in vivo viability of human blastocysts collapsed by laser pulse or osmotic shock prior to vitrification. *J Assist Reprod Genet.* 2011;28(4):355–61.
157. Cao S, et al. Retrospective clinical analysis of two artificial shrinkage methods applied prior to blastocyst vitrification on the outcome of frozen embryo transfer. *J Assist Reprod Genet.* 2014;31(5):577–81.
158. Van Landuyt L, et al. A prospective randomized controlled trial investigating the effect of artificial shrinkage (collapse) on the implantation potential of vitrified blastocysts. *Hum Reprod.* 2015;30(11):2509–18.
159. Min SH, et al. Forced collapse of the blastocoel enhances survival of cryotop vitrified bovine hatching/hatched blastocysts derived from in vitro fertilization and somatic cell nuclear transfer. *Cryobiology.* 2013;66(2):195–9.
160. Roth TL, Swanson WF, Wildt DE. Developmental competence of domestic cat embryos fertilized in vivo versus in vitro. *Biol Reprod.* 1994;51(3):441–51.
161. Enright BP, et al. Culture of in vitro produced bovine zygotes in vitro vs in vivo: Implications for early embryo development and quality. *Theriogenology.* 2000;54(5):659–73.
162. Rizos D, et al. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev.* 2002;61(2):234–48.
163. Mahmoudzadeh AR, et al. Optimization of a simple vitrification procedure for bovine embryos produced in vitro: Effect of developmental stage, two-step addition of cryoprotectant and sucrose dilution on embryonic survival. *J Reprod Fertil.* 1995;103(1):33–9.
164. Wurth YA, et al. Developmental potential of in vitro produced bovine embryos following cryopreservation and single-embryo transfer. *Theriogenology.* 1994;42:1275–84.
165. Reinders JMC. From embryo to a calf after embryo transfer, a comparison of in vivo and in vitro produced embryos. *Theriogenology.* 1995;43:306.
166. Agca Y, et al. Transfer of fresh and cryopreserved IVP bovine embryos: Normal calving, birth weight and gestation lengths. *Theriogenology.* 1998;50(1):147–62.
167. Sirisha K, et al. Cryopreservation of zona-free cloned buffalo (*Bubalus bubalis*) embryos: Slow freezing vs open-pulled straw vitrification. *Reprod Domest Anim.* 2013;48(4):538–44.
168. Zander-Fox D, Lane M, Hamilton H. Slow freezing and vitrification of mouse morula and early blastocysts. *J Assist Reprod Genet.* 2013;30(8):1091–8.
169. Check JH, et al. Fresh embryo transfer is more effective than frozen for donor oocyte recipients but not for donors. *Hum Reprod.* 2001;16(7):1403–8.
170. Nagy ZP, et al. Removal of lysed blastomeres from frozen-thawed embryos improves implantation and pregnancy rates in frozen embryo transfer cycles. *Fertil Steril.* 2005;84(6):1606–12.
171. Elliott TA, et al. Lysed cell removal promotes frozen-thawed embryo development. *Fertil Steril.* 2007;87(6):1444–9.
172. Jelinkova L, et al. Twin pregnancy after vitrification of 2-pronuclei human embryos. *Fertil Steril.* 2002;77(2):412–4.
173. Vanderzwalmen P, et al. Vitrification of human blastocysts with the hemi-straw carrier: Application of assisted hatching after thawing. *Hum Reprod.* 2003;18(7):1504–11.
174. Vanderzwalmen P, et al. Pregnancies after vitrification of human day 5 embryos. *Hum Reprod.* 1997;12(Suppl):98.
175. Mukaida T, et al. Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Hum Reprod.* 1998;13(10):2874–9.
176. Park SP, et al. Ultra-rapid freezing of human multipronuclear zygotes using electron microscope grids. *Hum Reprod.* 2000;15(8):1787–90.
177. Saito H, et al. Application of vitrification to human embryo freezing. *Gynecol Obstet Invest.* 2000;49(3):145–9.
178. Yokota Y, et al. Successful pregnancy following blastocyst vitrification: Case report. *Hum Reprod.* 2000;15(8):1802–3.
179. Yokota Y, et al. Birth of a healthy baby following vitrification of human blastocysts. *Fertil Steril.* 2001;75(5):1027–9.
180. Liebermann J, Tucker MJ. Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. *Reproduction.* 2002;124(4):483–9.
181. Reed ML, et al. Vitrification of human blastocysts using the cryoloop method: Successful clinical application and birth of offspring. *J Assist Reprod Genet.* 2002;19(6):304–6.
182. Son WY, et al. Ongoing twin pregnancy after vitrification of blastocysts produced by in-vitro matured oocytes retrieved from a woman with polycystic ovary syndrome: Case report. *Hum Reprod.* 2002;17(11):2963–6.
183. Isachenko V, et al. Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws. *Hum Reprod.* 2005;20(2):492–6.
184. Liebermann J, Tucker MJ. Vitrifying and warming of human oocytes, embryos, and blastocysts: Vitrification procedures as an alternative to conventional cryopreservation. *Methods Mol Biol.* 2004;254:345–64.
185. Zheng WT, et al. Comparison of the survival of human biopsied embryos after cryopreservation with four different methods using non-transferable embryos. *Hum Reprod.* 2005;20(6):1615–8.

186. Stehlík E, et al. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reprod Biomed Online*. 2005;11(1):53–7.
187. Balaban B, et al. A randomized controlled study of human day 3 embryo cryopreservation by slow freezing or Vitrification: Vitrification is associated with higher survival, metabolism and blastocyst formation. *Hum Reprod*. 2008;23(9):1976–82.
188. Rezazadeh Valojerdi M, et al. Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. *J Assist Reprod Genet*. 2009;26(6):347–54.
189. Son WY, et al. Comparison of survival rate of cleavage stage embryos produced from in vitro maturation cycles after slow freezing and after vitrification. *Fertil Steril*. 2009;92(3): 956–8.
190. Keskintepé L, et al. Vitrification of human embryos subjected to blastomere biopsy for pre-implantation genetic screening produces higher survival and pregnancy rates than slow freezing. *J Assist Reprod Genet*. 2009;26(11-12):629–35.
191. Wilding MG, et al. Human cleavage-stage embryo vitrification is comparable to slow-rate cryopreservation in cycles of assisted reproduction. *J Assist Reprod Genet*. 2010;27(9-10):549–54.
192. Sifer C, et al. Outcome of embryo vitrification compared to slow freezing process at early cleavage stages. Report of the first French birth. *Gynecol Obstet Fertil*. 2012;40(3):158–61.
193. AbdelHafez FF, et al. Slow freezing, vitrification and ultra-rapid freezing of human embryos: A systematic review and meta-analysis. *Reprod Biomed Online*. 2010;20(2):209–22.
194. Desai N, et al. Cryoloop vitrification of human day 3 cleavage-stage embryos: Post-vitrification development, pregnancy outcomes and live births. *Reprod Biomed Online*. 2007;14(2):208–13.
195. Hong SW, et al. Cryopreserved human blastocysts after vitrification result in excellent implantation and clinical pregnancy rates. *Fertil Steril*. 2009;92(6):2062–4.
196. Hiraoka K, Kinutani M, Kinutani K. Vitrification of human hatched blastocysts: A report of 4 cases. *J Reprod Med*. 2007;52(5): 413–5.
197. Stachecki JJ, et al. A new safe, simple and successful vitrification method for bovine and human blastocysts. *Reprod Biomed Online*. 2008;17(3):360–7.
198. Selman H, et al. Vitrification is a highly efficient method to cryopreserve human embryos in in vitro fertilization patients at high risk of developing ovarian hyperstimulation syndrome. *Fertil Steril*. 2009;91(4 Suppl):1611–3.
199. Hiraoka K, et al. Vitrified human day-7 blastocyst transfer: 11 cases. *Reprod Biomed Online*. 2008;17(5):689–94.
200. Wirleitner B, et al. The time aspect in storing vitrified blastocysts: Its impact on survival rate, implantation potential and babies born. *Hum Reprod*. 2013;28(11):2950–7.
201. Cobo A, et al. Six years' experience in ovum donation using vitrified oocytes: Report of cumulative outcomes, impact of storage time, and development of a predictive model for oocyte survival rate. *Fertil Steril*. 2015;104(6):1426–34.e8.
202. Achour R, et al. [Embryo vitrification: First Tunisian live birth following embryo vitrification and literature review]. *Tunis Med*. 2015;93(3):181–3.
203. Sparks AE. Human embryo cryopreservation-methods, timing, and other considerations for optimizing an embryo cryopreservation program. *Semin Reprod Med*. 2015;33(2):128–44.
204. Knopman JM, et al. Women with cancer undergoing ART for fertility preservation: A cohort study of their response to exogenous gonadotropins. *Fertil Steril*. 2009;91(4 Suppl):1476–8.
205. Grifo JA, Noyes N. Delivery rate using cryopreserved oocytes is comparable to conventional in vitro fertilization using fresh oocytes: Potential fertility preservation for female cancer patients. *Fertil Steril*. 2010;93(2):391–6.
206. Noyes N, et al. Oocyte cryopreservation as a fertility preservation measure for cancer patients. *Reprod Biomed Online*. 2010;23(3):323–33.
207. Lockwood G. Politics, ethics and economics: Oocyte cryopreservation in the UK. *Reprod Biomed Online*. 2003;6(2):151–3.
208. Bedoschi G, Oktay K. Current approach to fertility preservation by embryo cryopreservation. *Fertil Steril*. 2013;99(6):1496–502.
209. Cobo A, et al. New options in assisted reproduction technology: The cryotop method of oocyte vitrification. *Reprod Biomed Online*. 2008;17(1):68–72.
210. Nagy ZP, et al. The efficacy and safety of human oocyte vitrification. *Semin Reprod Med*. 2009;27(6):450–5.
211. Lin YH, et al. Combination of cabergoline and embryo cryopreservation after GnRH agonist triggering prevents OHSS in patients with extremely high estradiol levels—a retrospective study. *J Assist Reprod Genet*. 2013;30(6):753–9.
212. Imudia AN, et al. Elective cryopreservation of all embryos with subsequent cryothaw embryo transfer in patients at risk for ovarian hyperstimulation syndrome reduces the risk of adverse obstetric outcomes: A preliminary study. *Fertil Steril*. 2013;99(1):168–73.
213. Stoop D, et al. Offering excess oocyte aspiration and vitrification to patients undergoing stimulated artificial insemination cycles can reduce the multiple pregnancy risk and accumulate oocytes for later use. *Hum Reprod*. 2010;25(5):1213–8.
214. Joris H, et al. Reduced survival after human embryo biopsy and subsequent cryopreservation. *Hum Reprod*. 1999;14(11):2833–7.
215. Jericho H, et al. A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos. *Hum Reprod*. 2003;18(3):568–71.
216. Schoolcraft WB, Katz-Jaffe MG. Comprehensive chromosome screening of trophectoderm with vitrification facilitates elective single-embryo transfer for infertile women with advanced maternal age. *Fertil Steril*. 2013;100(3):615–9.
217. Grifo JA, et al. Single thawed euploid embryo transfer improves IVF pregnancy, miscarriage, and multiple gestation outcomes and has similar implantation rates as egg donation. *J Assist Reprod Genet*. 2013;30(2):259–64.
218. Schoolcraft WB, et al. Clinical application of comprehensive chromosomal screening at the blastocyst stage. *Fertil Steril*. 2010;94(5):1700–6.
219. Fragouli E, et al. Comprehensive chromosome screening of polar bodies and blastocysts from couples experiencing repeated implantation failure. *Fertil Steril*. 2010;94(3):875–87.
220. Schoolcraft WB, et al. Live birth outcome with trophectoderm biopsy, blastocyst vitrification, and single-nucleotide polymorphism microarray-based comprehensive chromosome screening in infertile patients. *Fertil Steril*. 2011;96(3):638–40.
221. Oakes MB, et al. A case of oocyte and embryo vitrification resulting in clinical pregnancy. *Fertil Steril*. 2008;90(5):2013 e5–8.
222. Peng W, Zhang J, Shu Y. Live birth after transfer of a twice-vitrified warmed blastocyst that had undergone trophectoderm biopsy. *Reprod Biomed Online*. 2011;22(3):299–302.
223. Greco E, et al. Successful implantation and live birth of a healthy boy after triple biopsy and double vitrification of oocyte-embryo-blastocyst. *Springerplus*. 2015;4:22.
224. Shapiro BS, et al. Clinical rationale for cryopreservation of entire embryo cohorts in lieu of fresh transfer. *Fertil Steril*. 2014;102(1):3–9.
225. Mohamed AM, et al. Live birth rate in fresh and frozen embryo transfer cycles in women with endometriosis. *Eur J Obstet Gynecol Reprod Biol*. 2011;156(2):177–80.
226. Shapiro BS, et al. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: A prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. *Fertil Steril*. 2011;96(2):344–8.
227. Cobo A, et al. Accumulation of oocytes: A new strategy for managing low-responder patients. *Reprod Biomed Online*. 2012;24(4): 424–32.
228. Vanderzwalmen P, et al. Blastocyst transfer after aseptic vitrification of zygotes: An approach to overcome An impaired uterine environment. *Reprod Biomed Online*. 2012;25(6):591–9.

229. Roy TK, et al. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. *Fertil Steril.* 2014;101(5):1294–301.
230. Chian RC, et al. Obstetric outcomes following vitrification of in vitro and in vivo matured oocytes. *Fertil Steril.* 2009;91(6):2391–8.
231. Noyes N, Porcu E, Borini A. Over 900 oocyte cryopreservation babies born with no apparent increase in congenital anomalies. *Reprod Biomed Online.* 2009;18(6):769–76.
232. Rama Raju GA, et al. Neonatal outcome after vitrified day 3 embryo transfers: A preliminary study. *Fertil Steril.* 2009;92(1):143–8.
233. Shu Y, Peng W, Zhang J. Pregnancy and live birth following the transfer of vitrified-warmed blastocysts derived from zona- and corona-cell-free oocytes. *Reprod Biomed Online.* 2010;21(4):527–32.
234. Wiklund M, et al. Obstetric outcomes after transfer of vitrified blastocysts. *Hum Reprod.* 2010;25(7):1699–707.
235. Shi W, et al. Perinatal and neonatal outcomes of 494 babies delivered from 972 vitrified embryo transfers. *Fertil Steril.* 2012;97(6):1338–42.
236. Chen Y, et al. Neonatal outcomes after the transfer of vitrified blastocysts: Closed versus open vitrification system. *Reprod Biol Endocrinol.* 2013;11:107.
237. Li Z, et al. Clinical outcomes following cryopreservation of blastocysts by vitrification or slow freezing: A population-based cohort study. *Hum Reprod.* 2014;29(12):2794–801.
238. Devine K, et al. Single vitrified blastocyst transfer maximizes live-born children per embryo while minimizing preterm birth. *Fertil Steril.* 2015;103(6):1454–60 e1.
239. Kong IK, et al. Comparison of open pulled straw (OPS) vs glass micropipette (GMP) vitrification in mouse blastocysts. *Theriogenology.* 2000;53(9):1817–26.
240. Isachenko V, Vajta A. Double cryopreservation of rat embryos at different developmental stages with identical vitrification protocol: The not properly understood phenomenon. *J Reprod Fertil.* 2000;26:Abstract series:10.
241. Tominaga K, Hamada Y. Gel-loading tips as container for vitrification of in vitro-produced bovine embryos. *J Reprod Dev.* 2001;47:259–265.
242. Liebermann J, et al. Blastocyst development after vitrification of multipronuclear zygotes using the flexipet denuding pipette. *Reprod Biomed Online.* 2002;4(2):146–50.
243. Cremades N, et al. Experimental vitrification of human compacted morulae and early blastocysts using fine diameter plastic micropipettes. *Hum Reprod.* 2004;19(2):300–5.
244. Hredzak R, et al. [Clinical experience with a modified method of human embryo vitrification]. Ceska gynekologie/Ceska Lekarska Spolecnost J. Ev. Purkyne. 2005;70(2):99–103.
245. Chen SU, et al. Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. *Hum Reprod.* 2001;16(11):2350–6.
246. Lopez-Bejar M, Lopez-Gatius F. Nonequilibrium cryopreservation of rabbit embryos using a modified (sealed) open pulled straw procedure. *Theriogenology.* 2002;58(8):1541–52.
247. Arav A. Vitrification of oocytes and embryos. In: New Trends in Embryo Transfer. Lauria A, Gandolfi F. (eds.). Portland Press: Cambridge. pp. 255–264, 1992.
248. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online.* 2006;12(6):779–96.
249. Vieira AD, et al. Calves born after open pulled straw vitrification of immature bovine oocytes. *Cryobiology.* 2002;45(1):91–4.
250. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol Reprod.* 1996;54(5):1059–69.
251. Hou YP, et al. Bovine oocytes vitrified by the open pulled straw method and used for somatic cell cloning supported development to term. *Theriogenology.* 2005;64(6):1381–91.
252. Booth PJ, et al. Full-term development of nuclear transfer calves produced from open-pulled straw (OPS) vitrified cytoplasts: Work in progress. *Theriogenology.* 1999;51(5):999–1006.
253. French AJ, et al. Viability of cloned bovine embryos following OPS vitrification. *Theriogenology.* 2002;57:413.
254. French AJ, et al. Generation of a¹ casein gene transgenic calves by nuclear transfer. *Biol Reprod.* 2003;68:240.
255. Peura TT, et al. No differences in sheep somatic cell nuclear transfer outcomes using serum-starved or actively growing donor granulosa cells. *Reprod Fertil Dev.* 2003;15(3):157–65.
256. Fujihira T, Kishida R, Fukui Y. Developmental capacity of vitrified immature porcine oocytes following ICSI: Effects of cytochalasin B and cryoprotectants. *Cryobiology.* 2004;49(3):286–90.
257. Fujihira T, Fukui NH. Relationship between equilibration time and the presence of cumulus cells, and effect of taxol treatment for vitrification of in vitro matured porcine oocytes. *Cryobiology.* 2005;51:339–343.
258. Kobayashi S, et al. Piglets produced by transfer of vitrified porcine embryos after stepwise dilution of cryoprotectants. *Cryobiology.* 1998;36(1):20–31.
259. Berthelot F, et al. Birth of piglets after OPS vitrification and transfer of compacted morula stage embryos with intact zona pellucida. *Reprod Nutr Dev.* 2001;41(3):267–72.
260. Men H, et al. Beneficial effects of serum supplementation during in vitro production of porcine embryos on their ability to survive cryopreservation by open pulled straw vitrification. *Theriogenology.* 2005;64(6):1340–9.
261. MacLellan LJ, et al. Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. *Theriogenology.* 2002;58(5):911–9.
262. Piltti K, et al. Live cubs born after transfer of OPS vitrified-warmed embryos in the farmed European polecat (*Mustela putorius*). *Theriogenology.* 2004;61(5):811–20.
263. Crichton EG, et al. Efficacy of porcine gonadotropins for repeated stimulation of ovarian activity for oocyte retrieval and in vitro embryo production and cryopreservation in Siberian tigers (*Panthera tigris altaica*). *Biol Reprod.* 2003;68(1):105–13.
264. Iwayama H, et al. Effects of cryodevice type and donors' sexual maturity on vitrification of minke whale (*Balaenoptera bonaerensis*) oocytes at germinal vesicle stage. *Zygote.* 2004;12(4):333–8.

Appendix: Embryo/blastocyst vitrification protocol

Vitrification

Materials

Equilibration Solution (ES) is a HEPES buffered medium, 7.5% (v/v) of each DMSO and ethylene glycol and 20% (v/v) serum protein substitute.

Vitrification Solution (VS) is a HEPES buffered medium, 15% (v/v) of each DMSO and ethylene glycol and 20% (v/v) serum protein substitute and 0.5 M sucrose.

Cryolock® Biodesign, Columbia

Procedures

1. Bring one vial of each ES and VS to room temperature (20°C–27°C) for at least 30 minutes prior to freezing embryos.
2. Fill the liquid nitrogen reservoir with liquid nitrogen.
3. Determine the number of embryos to be vitrified.
4. Label each Cryolock with necessary information.
5. Prepare four-well dish with 1.0 mL ES and 1.0 mL VS in each well.

6. Transfer the embryos to ES for 15 minutes.
7. Transfer the embryos to VS for 1 minute.
8. Load the embryos onto the Cryolock with a minimal volume.
9. Plunge the Cryolock into liquid nitrogen (cooling at a rate of $-12,000^{\circ}\text{C}/\text{minute}$).
10. Move the plunged Cryolock to the liquid nitrogen freezer for long-term storage.

Warming Materials

Thawing Solution (TS) is a HEPES buffered medium, 1.0 M sucrose and 20% (v/v) serum protein substitute.

Dilution Solution (DS) is a HEPES buffered medium, 0.5 M sucrose and 20% (v/v) serum protein substitute.

Washing Solution (WS) is a HEPES buffered medium and 20% (v/v) serum protein substitute.

Procedures

1. Bring one vial of each TS, DS, and WS to room temperature ($20^{\circ}\text{C} - 27^{\circ}\text{C}$) for at least 30 minutes prior to thawing embryos.
2. Fill the liquid nitrogen reservoir with liquid nitrogen.
3. Determine the number of embryos to be thawed.
4. Take the Cryolock out of the LN_2 and quickly transfer embryos into TS (3 mL at 37°C), where embryos should stay for 1 minute.
5. Transfer the embryos into 1.0 mL DS for 3 minutes at RT.
6. Transfer the embryos into 1.0 mL WS for 10 minutes at RT.
7. Transfer the embryos into pre-equilibrated culture medium.

MANAGING AN OOCYTE BANK

Ana Cobo, Pilar Alamá, José María De Los Santos, María José De Los Santos, and José Remohí

Introduction

Nowadays, the challenge of the cryopreservation, long-term storage, and successful implantation of the female gamete is feasible thanks to vitrification. There is a large population that is currently benefiting from oocyte banks, such as cancer patients who need an option for fertility preservation before undergoing potentially sterilizing treatment [1] or women who wish to delay their motherhood due to a variety of reasons [2, 3]. Oocyte cryo-storage brings additional advantages to assisted reproduction technology (ART) programs, being helpful in solving different clinical situations such as low-response patients [4], unpredictable availability of semen sample collection from the male partner, risk of suffering from ovarian hyperstimulation syndrome [5], or some other cases in which embryo transfer is not advisable [6]. Undoubtedly, ovum donation programs have also been major beneficiaries of egg banking. Oocyte cryo-storage is very useful for overcoming the most common drawbacks involved in ovum donation as currently applied, such as synchronization between donors and recipients, long waiting lists subject to the availability of a suitable donor, and, most important, the absence of a quarantine period.

In spite of its great value, oocyte cryo-storage has not been a valid option until relatively recently, due to the lack of successful methodologies. The reasons behind the long period of failures in attempts to cryopreserve oocytes are well identified. Among them, the size and shape of the female gamete are two significant reasons. The female gamete is the largest cell of the human body, with a large content of water, leading to a higher probability of ice formation during the cryopreservation process. Chilling injury, defined as irreversible damage to the cytoskeleton [7] and cell membranes [8], following exposure of cells to low temperatures from +15 to -5°C before the nucleation of ice is another major factor responsible for cell death during cryopreservation [9]. Ice crystal formation within the cytoplasm must be avoided at all costs in order to guarantee the survival and integrity of the cells when they are later thawed. Vitrification efficiently avoids chilling injury by direct passage from room temperatures to -196°C and so avoids ice formation [10]. Vitrification employs both high cooling rates and high cryoprotectant concentrations [11]. However, due to the potential toxicity of these compounds, the vitrification protocols have been modified in order to reduce damage. Additionally, efforts have also focused on increasing both the cooling and the warming rates in order to guarantee the viability of the cells [12, 13]. As a result, these days we count on several efficient approaches that are able to provide successful outcomes comparable to those achieved with fresh oocytes, thus making oocyte banking a reliable approach.

In this chapter, we will briefly review the clinical outcomes achieved with the use of vitrified oocytes in ovum donation, but we will primarily focus on the essential issues related to the management of the oocyte bank, including a description of the facilities, the equipment for storage, and liquid nitrogen (LN) supply. We will also evaluate the most relevant clinical aspects involved

in the management of the oocyte bank, such as donor selection, preparation of recipients, and the matching process.

Clinical outcome using an oocyte bank for ovum donation

Similar embryo development has been previously shown in embryos that originated from fresh versus vitrified oocytes in a sibling cohort study [14], whereas the clinical validation of using vitrified oocytes for egg donation was later demonstrated in a large randomized controlled clinical trial [15]. Comparable obstetric and perinatal outcomes of the babies conceived using vitrified versus fresh oocytes have been recently demonstrated in a large study involving more than 2000 infants, suggesting the harmlessness of the technology [16].

The use of cryo-stored oocytes in a large ovum donation program has been evaluated recently [17]. The overall survival rate analysed in this large series including over 40,000 vitrified oocytes was 92.6%. The possible effects of storage time on the survival rate and clinical outcome was calculated in different time categories from less than six months until over five years, showing no impact on either survival rate or clinical outcome [17]. We believe that this is very reassuring information since success after long-term storage guarantees the sustainability of the approach. The clinical, ongoing pregnancy and delivery rates were 55.0%, 45.3%, and 37.6%, respectively, thus confirming the consistency of the results as compared to our previous findings [15, 18]. The likelihood of having surplus embryos available for additional cryo-transfers was very high in this series due to the mean number of oocytes donated. The possibility of further cryo-transfers increased cumulative outcomes, and thus maximized the yield of a single-donation cycle, which is precisely what we show herein. The cumulative delivery rate per donation cycle increased to more than 70% after three cryo-transfers and rose to nearly 80% after five cryo-transfers. These results render the donation cycle highly efficient. This finding supports the previous observations we made about the absence of harmful effects of double vitrification (i.e. vitrified embryos developed from vitrified oocytes) [19]. The probability increases exponentially according to the number of oocytes consumed, and the patient can achieve a baby at any number of consumed oocytes with a probability of almost 100% when around three to four donation cycles are completed [17].

To date, we have notification of more than 16,000 babies born ($n = 16,739$) after above 40,000 ovum donation cycles with vitrified oocytes (41,042), involving nearly 500,000 vitrified oocytes ($n = 488,022$) in the Instituto Valenciano de Infertilidad (IVI) group (unpublished data), revealing the great scope of this approach. At present, more than 20,000 *in vitro* fertilization (IVF) cycles ($n = 20,229$) involving the use of own vitrified oocytes (~190,000 oocytes) have been performed at our centres (4384 babies born from whom we have notification), accounting for ~21,000 children born from vitrified oocytes in our group.

Logistics and technical aspects related to the oocyte bank

Facilities

In accordance with European Directive 2004/23/EC, ART laboratories including centres or clinics, along with banks of gametes, are considered tissue establishments and therefore are under the regulations and standards that were placed to prevent the transmission of infectious diseases of human tissues and cells.

Safety measures need to be implemented not only during procurement, testing, and processing but also during preservation, distribution, use, and, of course, storage. Here, we will describe some of the technical features that an oocyte bank has to meet in order to fulfil the European regulations and so be qualified in the four following aspects: design, installation, operation, and performance.

Regarding the facilities, one of the aspects to be qualified in is related to location, air quality, and construction materials.

Location of the storage room

From the practical point of view, the storage room with the LN tanks should be located close to the IVF laboratory so the cryopreserved oocytes can be easily, rapidly, and successfully transferred to the storage room and into the LN tanks.

Concerning distribution purposes, having your own oocyte bank will be logically easier for distribution and use. However, oocyte transport is also a feasible and a safe option that will be reviewed in this chapter.

As far as dimensions are concerned, the storage room should be designed to allocate a sufficient number of tanks to the storage of the expected number of samples. Some experts suggest calculating the space based on a linear increment within a 10-year plan basis or to have an off-site storage room in case of urgent need for extra space [20, 21].

Environmental variables

Although storage facilities might not need to strictly follow the same environmental criteria as procurement and processing facilities, it is recommended, at least for oocyte banking in vapour phase and semi-closed systems, to implement preventive measures in order to minimize bacterial and other airborne contaminations during storage. Such preventive measures can be implemented by installing high-efficiency particulate air filters within the air conditioning system to remove small particles (<0.3 mm); positive pressure could also be considered as an option.

Tissue establishments in Europe must achieve grade A-quality environmental air during procurement and processing; however, since fewer critical steps are performed in the storage areas, grade D-quality background air is acceptable.

The effects of volatile organic compounds (VOCs) on cryopreserved human oocytes and embryos have not yet been evaluated; therefore, it is difficult to assess the level of stringency in terms of VOC control in the storage room. Our recommendation would be to control and minimize VOCs by use of fixed or mobile versions of photo-catalytic oxidizing units or similar approaches.

With regards to temperature, even though room temperature (22°C–23°C) should be adequate, setting up the room under a cold temperature might help to minimize the LN evaporation and water condensation that can facilitate microbial growth. Another approach can be undertaken by setting up a humidity controller.

Moreover, low-level oxygen sensors and alarm systems in case of LN leaks have to be put in place for safety reasons. As a part of the clinic's general emergency plan, the storage facility should

also have generators or an uninterrupted power supply system in case of loss of electrical power.

Equipment

All our samples are cryopreserved by vitrification. This procedure, as currently performed, is entirely manually operated, making the use of any equipment to carry out the vitrification process itself unnecessary. The ease and efficiency of vitrification have brought about a turning point in the field of cryopreservation, making the whole process take no longer than 20 minutes (vitrification and warming) and involving very simple tools. However, the fact that the samples are vitrified and mostly contained in very low volumes represents a challenge for further handling, storage, and maintenance of the vitrified samples. Here, we describe the material and equipment needed for the proper storage of vitrified oocytes in our oocyte bank facilities.

Storage tank

The storage vessel can be traditional LN tanks or vapour tanks. In our oocyte bank facilities, we use vapour-phase storage tanks (CBS V1500; Custom Biogenic Systems, Bruce Township, MI) which contain an outer jacket with LN (Figure 23.1). This is responsible for cooling the storage area where the oocytes are maintained in a nitrogen gas atmosphere. The cold spreads from the vacuum-insulated jacket by convection and through vents in the storage compartment that expel the nitrogen vapour downwards to the bottom of the freezer, thus creating a flow of extremely cold air through the entire storage area (Figures 23.1 and 23.2). The exceptional uniformity of temperature allows the whole storage tank to be used, achieving temperatures below –180°C at the upper level and –195°C at the bottom. Samples can be manipulated in safe temperature ranges (–180°C) thanks to the working area located on top of the storage area, thus avoiding any risk of accidental warming (Figure 23.2). Figure 23.3 shows the disposition of samples in the storage area. Nearly 11,000 Cryotops can be stored in each tank. An additional advantage of this storage system is that the supply of LN can be programmable, although it also can be performed manually. We have demonstrated the effectiveness of this storage vessel as a strategy for preventing the risk of cross-contamination due to direct contact with the LN, showing comparable results between vapour-stored oocytes versus those stored in conventional LN tanks [22].

For periodical cleaning and due to the more complex and sophisticated nature of these tanks, we recommend regular maintenance, which forces the emptying of the vessels and the temporary location of the samples in a backup tank intended for that purpose. The backup tank must provide the same safe conditions as the storage tank. The emptying for cleaning and maintenance should be scheduled in advance and needs to be performed following strict standard operating protocols.

Construction, nitrogen supply, and gas pipes

The types of construction materials should be similar to those used in procurement and processing facilities, consisting of smooth surfaces and being easy to clean. Perhaps one of the most particular considerations to be undertaken with regard to contraction materials are that the floor should be resistant to large changes in temperature so that it will not easily crack as a result of LN spills.

In our facilities, there are three essential elements for the nitrogen supply to the storage tanks: firstly, a large-scale reservoir of LN (cistern with 2400 L capacity able to supply LN to approximately 10 CBS V1500 vapour tanks) located outside the

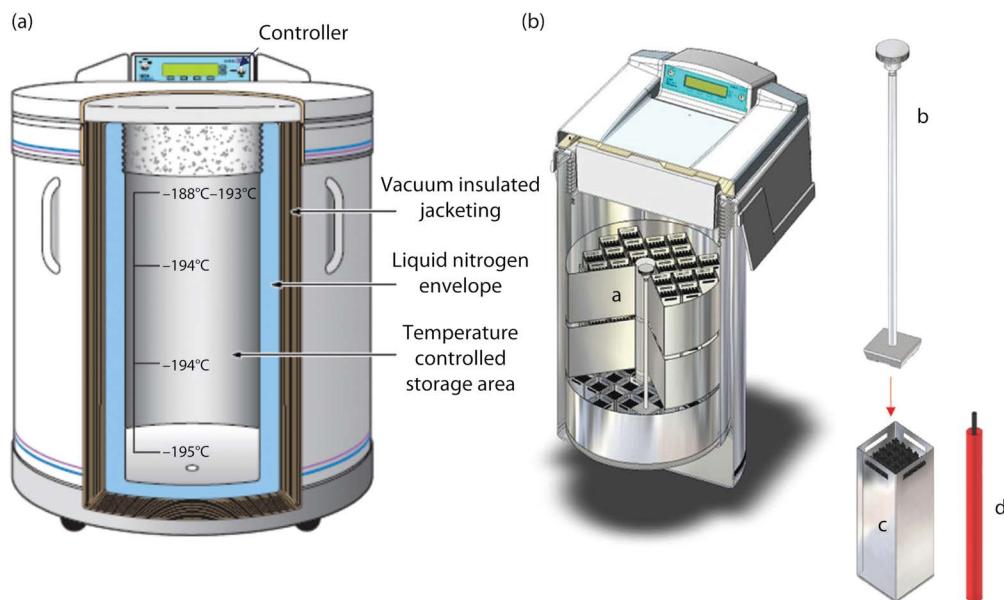


FIGURE 23.1 (A) Diagram to illustrate the inside of the tank, showing the jacket with liquid nitrogen (LN) and the vapour area for storage. (B) (a) Three storage levels assembled on a rotating carousel. (b) Retrieval tool to place and retrieve the canister (c). (d) Goblet containing the samples that are placed into the canisters.

building (Figure 23.4); secondly, a pressurized tank fed by the reservoir; and thirdly, vapour storage tanks that receive supply from the pressurized tank. The circuit is controlled by an automated, programmable system (Simatic Siemens PLC HMI, Nürnberg, Germany). The system is able to control a number of adjustable parameters, such as minimum and maximum permissible levels, pressure of LN filling, and low-level and overfilling alarms.

The conduction system for LN should be completely insulated to avoid loss of temperature and excess condensation and to minimize the evaporation of LN during refilling manoeuvres.

Additionally, individual valves allow the influx of LN into the jackets of the storage tanks. To prevent the impurities that LN may contain entering the storage tank, the use of a pre-filter is strongly recommended, as the presence of “debris” could cause serious problems to the valves of the storage tank (Figure 23.4c).

Nitrogen supply for the vitrification process

With the aim of purifying the LN used during the vitrification process, a specific ceramic filter is coupled to the pressurized tank (Figure 23.5). The Ceralin online filter (Air Liquide



FIGURE 23.2 (a) Working area of the vapour tank showing a storage canister (A arrow) and the vitrification rack (B arrow) at the time of storing oocytes. (b) Display showing the temperature while manipulating the oocytes (-184°C). (c) Storage room.

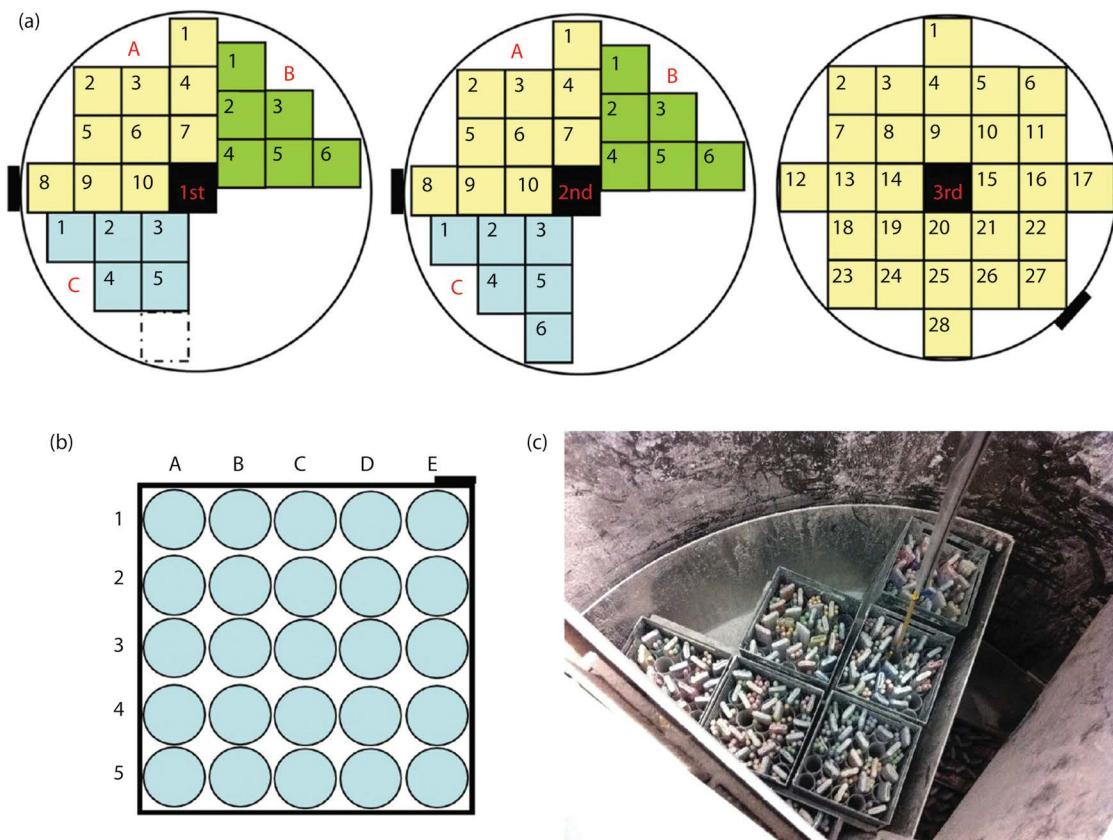


FIGURE 23.3 (a) Schematic drawing showing the arrangement and number of canisters for storage. Each canister is divided into 25 individual alphanumeric positions (b) for a total of 1800 positions in each tank. Each position can hold up to six Cryotops (10,800 Cryotops per bank). (c) Detail of some stored samples showing the canisters containing the goblets and Cryotops. Each goblet contains samples from individual donors. Placing samples from different patients in the same goblet is not allowed.

Medicinal, Paris, France) consists of a 0.1 µm ceramic membrane in accordance with US Food and Drug Administration Guidelines on Aseptic Processing (1987) [23]. The Ceralin online filter consists of two elements of liquid filtration connected in series and inserted into a section of the vacuum transfer line. The ceramic membrane is made from multiple layers formed into a multi-channel element. It is housed in a vacuum-insulated pipe, itself installed close to the end-use point. During operation, LN flows through the filter and over the ceramic membrane. The result is high-purity LN with a bacteria count of less than one colony-forming unit (CFU)/L gas. Additionally, the large filtration area of the membrane and low level of contamination of LN means it is likely to be several decades before filter saturation. Periodic sampling for microbial assessment is needed.

Temperature monitoring system during storage

Vitrified samples, especially those loaded in minimum volume in the vitrification device, are extremely sensitive to any change in temperature. For this reason, a temperature monitoring system is strongly advised as a part of the routine quality control (QC) of the cryo-lab. In our facilities, we use a system that allows continuous monitoring of the temperature of every storage tank in our unit (DataCare, ControlTemp, Barcelona, Spain). The system is able to provide numeric and graphic records (Figure 23.6) and display alarms in real time with updates every second. A record of incidents occurring during the alarm can also be easily assessed,

differentiating between active alarms or alarms that were active but are no longer in that state. In case of an alarm, the system sends alerts and warning messages to authorized personnel.

Safety during handling of LN

All safety measures for secure handling of LN must be observed. All laboratory personnel, especially embryologists/technicians in charge of the bank, vitrification, and all the related procedures must be aware of the Material Safety Data Sheet for LN and should be informed of the potential hazards of its use. The banking area should be located in a well-ventilated room. The measurement of oxygen levels is highly advisable due to high concentrations of nitrogen potentially reducing the breathable oxygen in the air. Approved personal protective equipment for eyes, cryogenic gloves, lab coats, closed-toe shoes, and long pants are mandatory.

Management of donors and recipients

Egg donor selection

Spanish assisted reproduction law is based on legislation that was passed in November 1988 (Law 35/1988) [24]. Although some countries already had regulations on or recommendations for ART at that time, Spain was the first country to create a specific law to cover this area of medicine. Royal Decree 412/1996 and Ministerial Order of March 25, 1996, established donor requirements, as well as mandatory standard screening procedures, to



FIGURE 23.4 (a) Reservoir tank for liquid nitrogen (LN) storage (Air Liquide, Madrid, Spain). (b) Detail of the touchscreen controller of the system showing the scheme for the filling of the pressurized tank. The filling of the pressurized tank (Apollo 350, Cryotherm, Kirchen (Sieg), Germany) begins at -130°C and is controlled by the system by actuating three solenoids (V1, V2, and V3). All the LN-phase gas coming from the reservoir tank via pipe A is disposed of in order to ensure that the pressurized tank is filled with liquid-phase nitrogen. The LN fills the pressurized tank (nurse tank) when the pre-set temperature is reached. The excess pressure generated during the filling phase is removed via pipe B. The valves automatically close when the filling is completed. LN is supplied to the vapour storage tanks via specific pipe C. In case of failure, the system can be handled manually by the action of the manual solenoids V4, V5, V6, and V7. (c) (1) Pipe with insulating coating for LN; (2) online wire mesh pre-filter; (3) entry valve for each tank. (d) Pressurized (nurse) tank. The arrow shows the ceramic filter Ceralin online.

rule out the transmission of genetic, hereditary, or infectious diseases [25]. In 2006, a new Spanish Law on Assisted Reproduction was approved (Law 14/2006) [26], which determined requirements for gamete and embryo use and regulations on financial compensation. A new Royal Decree 9/2014 established quality and safety standards for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells.

The following are the most important topics included in Spanish law on egg donation:

- Donation of human gametes is a formal, confidential contract between the donor and the reproductive medicine centre. Identities of donors must remain anonymous.
- The donation cannot be revoked.
- The maximum number of children generated from a single donor's gametes should not exceed six.

To be accepted as egg donors, women must be aged between 18 and 35 years and be healthy. The following steps are necessary to be admitted as an egg donor in our clinics:

- *Medical history:* During the first visit, an interview is conducted to complete the family and personal history.
- *Psychological screening:* Psychological evaluation and counselling by a qualified mental health professional.

The donor will be asked to speak with a psychologist to ensure that she fully understands the benefits and risks of egg donation and is properly motivated to become a donor.

- *Gynaecological examination:* Evaluation of the donor's menstrual cycles and vaginal ultrasound is needed to examine ovaries, count antral follicles, and to ensure that there is no pathology in her ovaries. At the same time, body mass index is calculated.
- *Medical screening:* This involves testing for blood type, Rh factor, antibody screening, complete blood cell count, haemostasis, biochemistry, and infectious disease screening, such as HIV, hepatitis C virus (HCV), and syphilis.
- *Genetic screening:* Blood tests for karyotype and carrier screening tests for severe recessive and X-linked childhood diseases based on next-generation sequencing (NGS) are conducted

To begin the egg donation cycle, an oral contraceptive pill is taken for a maximum of 21 days, which starts on days 1 or 2 of the menses of the previous cycle [27]. After a five-day washout period following taking the last pill, donors start their stimulation protocol with 150–225 IU of recombinant follicle-stimulating hormone (FSH), and 225 IU of highly purified human menopausal gonadotropin (HP-hMG), or 150–225 IU of recombinant FSH

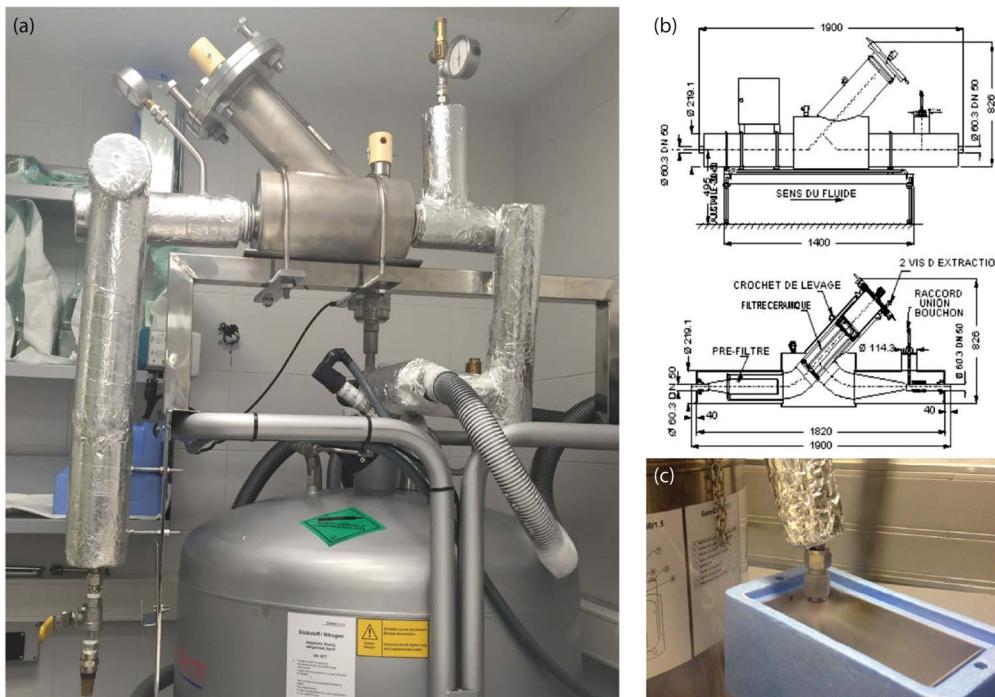


FIGURE 23.5 (a) Ceralin online filter (Air Liquide Medicinal, France). (b) Schematic illustration. (c) Collection of filtered liquid nitrogen (LN) in a sterile container used for vitrification.

plus 75 IU HP-hMG. Medroxyprogesterone acetate is administered orally as a single daily dose throughout stimulation until trigger day for pituitary suppression [28]. Egg donors are monitored regularly during FSH injections to measure follicle growth and to ensure it is within an appropriate range. Vaginal sonograms and blood tests are used to monitor follicle growth. Once follicles have matured enough for retrieval, a single dose of

gonadotropin-releasing hormone (GnRH) agonist is administered to trigger final oocyte maturation. Transvaginal oocyte retrieval takes place 36 hours after GnRH agonist administration. Donors receive light intravenous sedation for the egg retrieval procedure to ensure their comfort, and they rest for two hours at the clinic until they are discharged. In some cases, a post-retrieval vaginal scan is scheduled two to three days following egg retrieval [29].

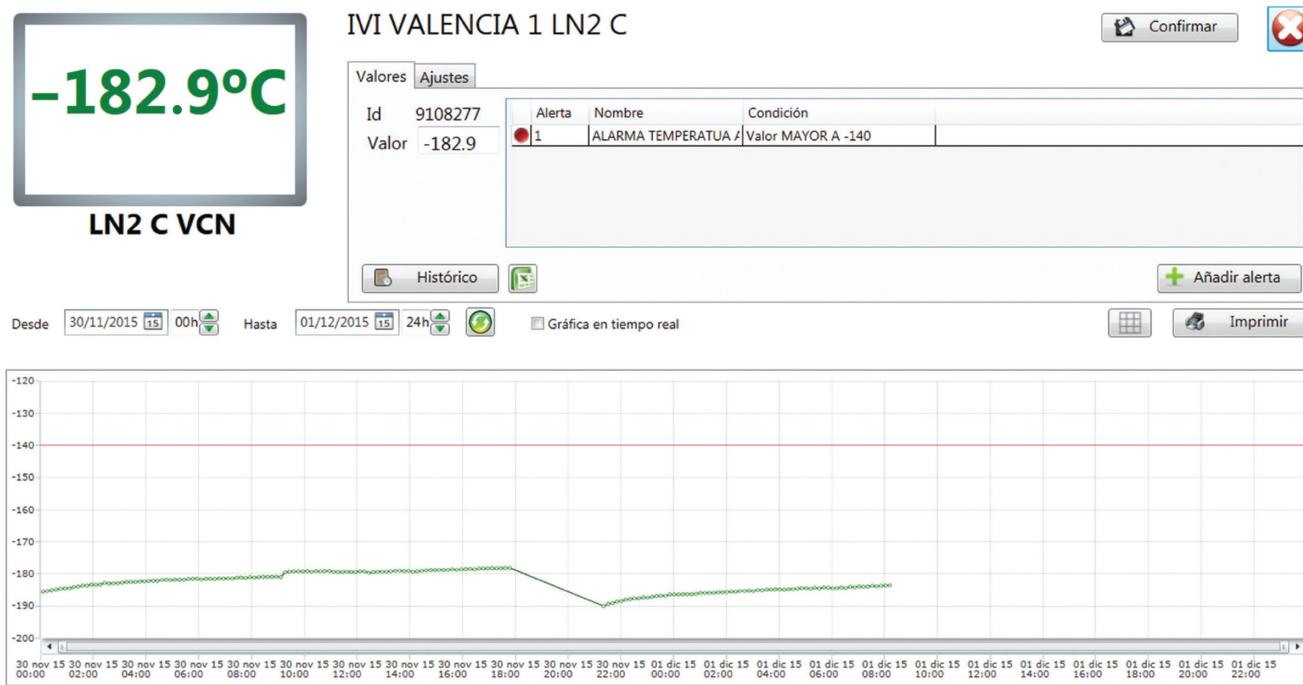


FIGURE 23.6 Datalogger graphic representation of temperature measurement over a time period of one vapour storage tank.

Oocyte recipients

Oocyte recipients enter our egg donation program for one of the following main diagnoses: premature ovarian failure/menopause; failure to achieve pregnancy after at least three cycles of assisted reproduction techniques; genetic or chromosomal disorders; low response to controlled ovarian hyperstimulation; or recurrent miscarriages.

The vast majority of oocyte recipients undergo hormone-replacement therapy (HRT). In patients with ovarian function, depot GnRH agonist is administered in the mid-luteal phase of their cycle, or GnRH antagonist is administered daily with menstruation for five days. HRT is initiated on days 1–3 of the following cycle with oral oestradiol valerate or an oestradiol transdermal patch [30–32]. Recipients without ovarian function are submitted to the same endometrial preparation protocol but are not administered a depot GnRH agonist. On days 15 or 16 of HRT, a transvaginal ultrasound is performed to measure endometrial thickness, and serum E2 and progesterone levels are tested. Most recipients are ready to receive embryos within two to three weeks of starting HRT, although administration of oestradiol valerate can be maintained for a maximum of 50 days until a suitable donation becomes available. Micronized progesterone (800 mg/day vaginally) is initiated on the day of oocyte donation, and embryos are transferred in the blastocyst stage. Progesterone levels are measured on the day of embryo transfer and luteal phase supplementation is adjusted according to progesterone levels [33]. The recipient continues taking oestrogen and progesterone with a positive pregnancy test, and these hormonal supplements are then continued through 12 weeks of pregnancy. Before treatment begins, the recipient undergoes preliminary testing. This assessment phase includes infectious disease screening (e.g. HIV, HCV, and syphilis), blood type, and Rh factor analysis for both parents and TRH. In women older than 45 years, a recent mammogram, full blood cell count, coagulation tests, and blood biochemistry may also be required.

To help the donor team select an egg donor, recipients will be asked to complete a form regarding their physical characteristics, such as hair colour, weight, height, eye colour, and biometrics (facial similarity study), among other traits.

It is advisable to collect a sperm sample if the partner lives far from the clinic.

Ovum donation synchronization

We consider many different factors during donor selection: we take into account race, reproductive history, and the physical characteristics that match those of the female partner, and we match blood type and genetic carrier screening. We call matching the time when we select a donor for a recipient after considering all the aforementioned factors.

The timing for the matching procedure has been improved in the last years thanks to the establishment of egg banking. However, it is important to note that in our current practice, we conduct donations both with fresh and vitrified oocytes, as long as fresh donations are still allowed in our country. Whether to conduct one strategy or another depends on different circumstances related to the availability of oocytes and the needs of the recipient.

Before introducing vitrification into our egg donation program, the numbers of donors and recipients in the clinic are determined at the time of matching: if there are many donors, the matching between donors and recipients is done on the day

of the donor's pickup. This means that sometimes recipients are on the waiting list for so many days that they may start bleeding. If, however, the clinic has very few donors needed for special considerations, then donors and recipients are synchronized. This means that recipients and donors start with ovarian stimulation (donors) and HRT (recipients) at the same time. Donor's stimulation may generate certain drawbacks as cycle cancellations due to different causes such as bleeding, etc., or fewer oocytes than expected). Consequently, the date for the donation is only indicative in these cases, therefore cannot be officially scheduled [18]. The likelihood of this happening underlines the importance of having a large egg donor bank with the availability of a large and varied number of stored oocytes that meet different characteristics.

In addition to the previous difficulties, about 65% of our recipients come from foreign countries. As such, compatibility issues, from a medical viewpoint, and the logistics of the process need to be considered.

As our usual medical practice now has an egg bank, the time of matching the donor and the recipient depends on different aspects, such as if recipients need specific characteristics or have requested a specific date for embryo transfer.

- Recipients who need specific characteristics: blood type (O negative, AB negative), specific race, screening for specific genetic diseases, or partners who would like to have another baby with the same donor as they had before:
 - First, we use our donor selection database and select one donor or two with the required characteristics. Sometimes there will be donors under stimulation with the required characteristics, and sometimes we call them to return to our clinic.
 - Second, all the oocytes obtained during pickup are vitrified for the recipient.
 - Finally, the recipient chooses the best time to schedule embryo transfer, and we provide them with instructions to begin HRT depending on embryo transfer.
- Recipients who do not need specific characteristics:
 - Recipients have a date for embryo transfer.
 - First, we make a reservation of oocytes from our egg donor bank.
 - Second, the recipient begins HRT depending on embryo transfer.
 - Finally, we have two options:
 - We use fresh oocytes when we have a donor pickup scheduled on the same date as the donation (with the same characteristics as the partner). The reservation of stored oocytes is cancelled in this case.
 - We use vitrified oocytes.
 - Recipients who do not have a date for embryo transfer:
 - The recipient begins HRT and remains on the waiting list.
 - We use fresh oocytes for the egg donation if we have scheduled a fresh donor pickup.
 - If the recipient stays on the waiting list longer than 20–25 days, we use oocytes from the egg donor bank.

We have created software that allows us to manage the ovum donation program, including all the relevant information about the donors. This application includes donors currently undergoing stimulation, donors with vitrified oocytes, and vitrified oocytes

The screenshot shows the IVI Matching Egg Donation software interface. At the top, it displays 'IVI Madrid' and 'IVI Valencia'. The main search area has two sections: 'PATIENT, TEST' and 'PARTNER, TEST'. Both sections include fields for Height (m), Weight (kg), BMI, Build, Ethnic background, and Relevant characteristics. Below this, there is a section titled 'Please select search characteristics' with dropdown menus for Skin colour, Eye colour, Hair colour, Hair texture, and other demographic details. On the right side, there are buttons for 'National only', 'Proven fertility', 'Fragile X', 'Cystic fibrosis', and 'CGT'. A large table below lists 'List of vitrified oocytes:14', 'List of ongoing fresh oocytes:1', 'List of fresh oocytes non-initiated:105', and a summary row. The table columns include Treatment, Protocol #, CHN, Full Name, Left, No. rcpt, No.ooc reserv, No. cop, Start age, Cent., Puncture date, Screening of monogenic, V., Prio., Cmts., and various icons for tracking. At the bottom, there are buttons for 'BCO', 'SEND ALERT MAIL', 'SQL', and 'Args'.

FIGURE 23.7 Matching sheet for donors and recipients. (Equipo IVI S.L.©)

located at different IVI facilities. The software also counts on a matching application that provides a list of the best possible donors after the introduction of recipients' characteristics, including phenotype, blood type, and other special features (Figure 23.7).

QC in the oocyte bank

The cryo-lab, including the bank, is part of the IVF lab, and, as such, it must be subjected to strict QC. In general, the same QC parameters for controlling the IVF lab are useful for the cryo-lab as well [34]. Accordingly, the cryo-lab needs to monitor and document the temperature, pH, osmolarity, and culture media, including vitrification solutions. The temperature of the storage tanks needs to be strictly controlled (Figure 23.6).

On the other hand, unlike other laboratory procedures, vitrification as currently performed is an entirely hand-operated procedure, for which outcomes are usually highly dependent on the embryologist/technician. Thus, in order to ensure efficiency, it should be performed only by highly skilled professionals who have undergone a long learning curve. Therefore, an adequate learning curve is also one of the most important requirements when performing vitrification that requires close attention. Our training program has produced satisfactory results since the introduction of vitrification in our clinical setting. It consists of different phases that gradually increase in difficulty. To pass to the next level, trainees must acquire the necessary skills as well as achieve a pre-set survival rate. Additionally, dynamic database

management analysis is routinely performed in order to monitor the maintenance of competence. Periodic analysis of success rates per operator is strongly advised. Figure 23.8 shows survival and clinical pregnancy rates per technician performing the vitrification and warming procedures.

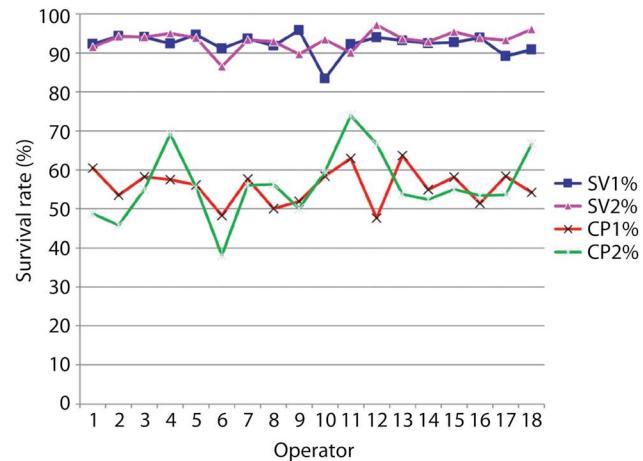


FIGURE 23.8 Survival and clinical outcomes according to the operator. SV1% and CP1%, survival and clinical pregnancy rates for the person doing the vitrification procedure; SV2% and CP2%, survival and clinical pregnancy rates for the person doing the warming procedure.

References

1. Cobo A, Domingo J, Pérez S, Crespo J, Remohi J, Pellicer A. Vitrification, an effective new approach to oocyte banking in healthy women, could be applied in cancer patients to preserve their fertility. *Clin Transl Oncol.* 2008;10:268–73.
2. Dondorp WJ, De Wert GM. Fertility preservation for healthy women: Ethical aspects. *Hum Reprod.* 2009;24(8):1779–85.
3. Garcia-Velasco JA, Domingo J, Cobo A, Martínez M, Carmona L, Pellicer A. Five years' experience using oocyte vitrification to preserve fertility for medical and nonmedical indications. *Fertil Steril.* 2013;99(7):1994–9.
4. Cobo A, Garrido N, Crespo J, Jose R, Pellicer A. Accumulation of oocytes: A new strategy for managing low-responder patients. *Reprod Biomed Online.* 2012;24(4):424–32.
5. Herrero L, Pareja S, Losada C, Cobo AC, Pellicer A, García-Velasco JA. Avoiding the use of human chorionic gonadotropin combined with oocyte vitrification and GnRH agonist triggering versus coasting: A new strategy to avoid ovarian hyperstimulation syndrome. *Fertil Steril.* 2011;95(3):1137–40.
6. Herrero L, Pareja S, Aragones M, Cobo A, Bronet F, García-Velasco JA. Oocyte versus embryo vitrification for delayed embryo transfer: An observational study. *Reprod Biomed Online.* 2014;29(5):567–72.
7. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril.* 1990;54(1):102–8.
8. Ghetler Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Hum Reprod.* 2005;20(12):3385–9.
9. Watson PF, Morris GJ. Cold shock injury in animal cells. *Symp Soc Exp Biol.* 1987;41:311–40.
10. Liebermann J, Dietl J, Vanderzwalmen P, Tucker MJ. Recent developments in human oocyte, embryo and blastocyst vitrification: Where are we now? *Reprod Biomed Online.* 2003;7(6):623–33.
11. Vajta G, Kuwayama M. Improving cryopreservation systems. *Theriogenology.* 2006;65(1):236–44.
12. Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reprod Biomed Online.* 2005;11(5):608–14.
13. Seki S, Mazur P. Effect of warming rate on the survival of vitrified mouse oocytes and on the recrystallization of intracellular ice. *Biol Reprod.* 2008;79(4):727–37.
14. Cobo A, Kuwayama M, Perez S, Ruiz A, Pellicer A, Remohi J. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the cryotop method. *Fertil Steril.* 2008;89(6):1657–64.
15. Cobo A, Meseguer M, Remohi J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: A prospective, randomized, controlled, clinical trial. *Hum Reprod.* 2010;25(9):2239–46.
16. Cobo A, Serra V, Garrido N, Olmo I, Pellicer A, Remohi J. Obstetric and perinatal outcome of babies born from vitrified oocytes. *Fertil Steril.* 2014;102(4):1006–15.e4.
17. Cobo A, Garrido N, Pellicer A, Remohi J. Six years' experience in ovum donation using vitrified oocytes: Report of cumulative outcomes, impact of storage time, and development of a predictive model for oocyte survival rate. *Fertil Steril.* 2015;104(6):1426–34.e1–8.
18. Cobo A, Remohi J, Chang CC, Nagy ZP. Oocyte cryopreservation for donor egg banking. *Reprod Biomed Online.* 2011;23(3):341–6.
19. Cobo A, De Los Santos JM, Castelló D, Pellicer A, Remohi J. Effect of re-vitrification of embryos achieved following oocyte vitrification on the new born rate. *Fertil Steril.* 2011;96(3 Suppl):S73.
20. Vajta G, Reichart A. Designing and operating cryopreservation facilities. In: A Practical Guide of Setting up an IVF Lab, Embryo, Culture Systems and Running the Unit. Varghese AC, Sjöblom P, Jayaprakasan K (eds.). New Delhi, India: Jaypee Brothers Ltd, pp. 54–9, 2013.
21. EDQM, Council of Europe. Guide of the quality and safety of tissues and cells for human application, 2nd edition [database on the Internet]. 2015. Available from: <https://www.edqm.eu/en/guide-to-the-quality-and-safety-of-tissues-and-cells-for-human-application>
22. Cobo A, Romero JL, Perez S, de los Santos MJ, Meseguer M, Remohi J. Storage of human oocytes in the vapor phase of nitrogen. *Fertil Steril.* 2010;94(5):1903–7.
23. Cobo A, Castelló D, Weiss B, Vivier C, De la Macorra A, Kramp F. Highest liquid nitrogen quality for vitrification process: Micro bacteriological filtration of LN2. 16th World Congress on In vitro Fertilization; 6th World Congress on In vitro Maturation, Tokyo, Japan. Abstract book. 2011; 286.
24. Disposiciones generales Ley 35/1988, de 22 de Noviembre, sobre Técnicas de Reproducción Asistida. 1988; 33373–8.
25. Decreto R. Donación gametas. RD 412/1996:11253–6.
26. LEY 14/2006, de 26 de mayo, sobre Técnicas de Reproducción Humana Asistida. 2006; 19947–56.
27. Remohi J, Vidal A, Pellicer A. Oocyte donation in low responders to conventional ovarian stimulation for *in vitro* fertilization. *Fertil Steril.* 1993;59(6):1208–15.
28. Giles J, Alama P, Gamiz P, Vidal C, Badia P, Pellicer A, Bosch E. Medroxyprogesterone acetate is a useful alternative to a gonadotropin-releasing hormone antagonist in oocyte donation: A randomized, controlled trial. *Fertil Steril.* 2021;116(2):404–412.
29. Remohi J, Gartner B, Gallardo E, Yalil S, Simon C, Pellicer A. Pregnancy and birth rates after oocyte donation. *Fertil Steril.* 1997;67(4):717–23.
30. Remohi J, Gutierrez A, Cano F, Ruiz A, Simon C, Pellicer A. Long oestradiol replacement in an oocyte donation programme. *Hum Reprod.* 1995;10(6):1387–91.
31. Soares SR, Troncoso C, Bosch E et al. Age and uterine receptiveness: Predicting the outcome of oocyte donation cycles. *J Clin Endocrinol Metab.* 2005;90(7):4399–404.
32. Soares SR, Velasco JA, Fernandez M, Bosch E, Remohi J, Pellicer A et al. Clinical factors affecting endometrial receptiveness in oocyte donation cycles. *Fertil Steril.* 2008;89(3):491–501.
33. Labarta E, Mariani G, Paolelli S, Rodriguez-Varela C, Vidal C, Giles J, et al. Impact of low serum progesterone levels on the day of embryo transfer on pregnancy outcome: A prospective cohort study in artificial cycles with vaginal progesterone. *Hum Reprod.* 2021 Feb 18;36(3):683–92.
34. Mortimer D, Pool TR, Cohen J. Introduction to quality management in assisted reproductive technology symposium. *Reprod Biomed Online.* 2014;28(5):533–4.

24

SEVERE MALE FACTOR INFERTILITY

Genetic Consequences and Recommendations for Genetic Testing

Katrien Stouffs

Overview

Infertility associated with a severe male factor such as oligoastheno-teratozoospermia (OAT) or azoospermia may be of genetic origin. This means that either the number or the structure of the chromosomes may be aberrant or a gene defect may be present. By knowing the underlying molecular cause of the fertility problems, appropriate genetic counselling can be offered to the patient, his partner, and his family whenever indicated. The role of genetic counselling in case of infertility has increased since the advent of assisted reproduction technology (ART) in general, and certainly since the introduction of intracytoplasmic sperm injection (ICSI), offering the possibility to have children to men with almost no spermatozoa [1–3]. Based on the available data, today a number of genetic tests should also be performed in case of infertility in an otherwise healthy male. For years, the main diagnostic tests have been the analysis of the karyotype in peripheral lymphocytes, a search for the presence of a Yq11 deletion on the long arm of the Y chromosome, and/or an analysis of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene in couples in which the male partner has congenital bilateral absence of the vas deferens (CBAVD). More specific genetic investigations can be done if indicated, especially when the azoospermia or oligozoospermia is part of a more complex disease or syndrome. Although currently not offered on a routine basis for patients with azoospermia or oligozoospermia, next-generation sequencing will be implemented in the near future.

Genetic causes of azoospermia and oligozoospermia

Chromosomal aberrations

It has been known for decennia that the presence of an extra X chromosome in males, resulting in a 47,XXY karyotype, causes Klinefelter syndrome, with testicular atrophy and non-obstructive azoospermia as main features [4, 5]. Since then, many chromosomal studies have been performed in series of infertile males, and the conclusions drawn from these studies are that constitutional chromosomal aberrations increase as sperm counts decrease.

From these studies, it is also clear that the incidence of numerical sex chromosomal aberrations such as 47,XXY and 47,XYY is proportionally higher in males with azoospermia than in males with oligozoospermia, whereas structural chromosomal aberrations of autosomes such as Robertsonian (Figure 24.1a) and reciprocal (Figure 24.1b) translocations are proportionally more frequent in oligozoospermic males (Table 24.1) [6–8].

In azoospermic males, it is also possible to find a 46,XX karyotype. In roughly 90% of these Klinefelter-like males the *SRY*

gene, normally located close to the pseudo-autosomal region of the short arm of the Y chromosome, is now, due to a crossing-over event during meiosis, present in that same region on one of the X chromosomes [9–11]. The *SRY* gene, referring to the sex-determining region of the Y chromosome, has to be expressed to induce the sexual development of an embryo towards a male phenotype [12]. In the remaining 10% of XX males, most probably other genes with functions in sexual development are involved. Spermatogenesis seems to be absent in these XX males, whereas in apparently non-mosaic Klinefelter patients sometimes a few spermatozoa can be found in testicular tissue. This can be explained by the absence of the long-arm of the Y chromosome containing the azoospermia factor (AZF) regions in XX males. Spermatozoa obtained from Klinefelter patients have been used in ICSI procedures, and healthy as well as a few 47,XXX children have been born (reviewed in Fullerton et al. [13]).

Microdeletions on the long-arm of the Y chromosome (Yq11)

The first azoospermic male patients in whom a deletion in the q11 region of the long-arm of the Y chromosome (Yq11) was linked to their infertility, were identified through conventional cytogenetic analysis [14]. At that time, the concept of the AZF region, the region lacking factors (genes) necessary for spermatogenesis due to a deletion, was introduced. Since that time, the structure of the Y chromosome, consisting of the gene-containing euchromatic parts (Yp and Yq11) and the polymorphic heterochromatic parts (Yq12), has been studied in detail using more sensitive molecular techniques. These have also helped to define the AZF region better. In fact, the AZF region consists of three subregions: AZFa, AZFb, and AZFc. Deletions in these subregions are most of the time not readily detectable by cytogenetic analysis. The most currently used techniques to reveal detailed information on the presence of deletions in this region are a polymerase chain reaction investigation with in-house developed primer sets or a commercially available kit. In the current guidelines, a two-step approach is recommended. First, the three subregions must be investigated through a multiplex PCR analysis. If a deletion is detected, an extension analysis must be performed to confirm the presence of a deletion and to investigate its size [15]. Also, massive parallel sequencing technologies are currently being applied for the detection of Yq microdeletions. Nevertheless, the investigation of the Y chromosome remains challenging due to the repetitive sequences located on the Y chromosome. In the late 1990s, multiple studies were performed to investigate the frequency of Yq microdeletions. These studies showed a prevalence of around 7.4% of Yq microdeletions, and again the prevalence is higher in azoospermic (9.7%) than in oligozoospermic (6.0%) males [16]. In most patients, the deletions span the AZFb and/or AZFc regions, whereas in a small number the AZFa region is deleted. Most

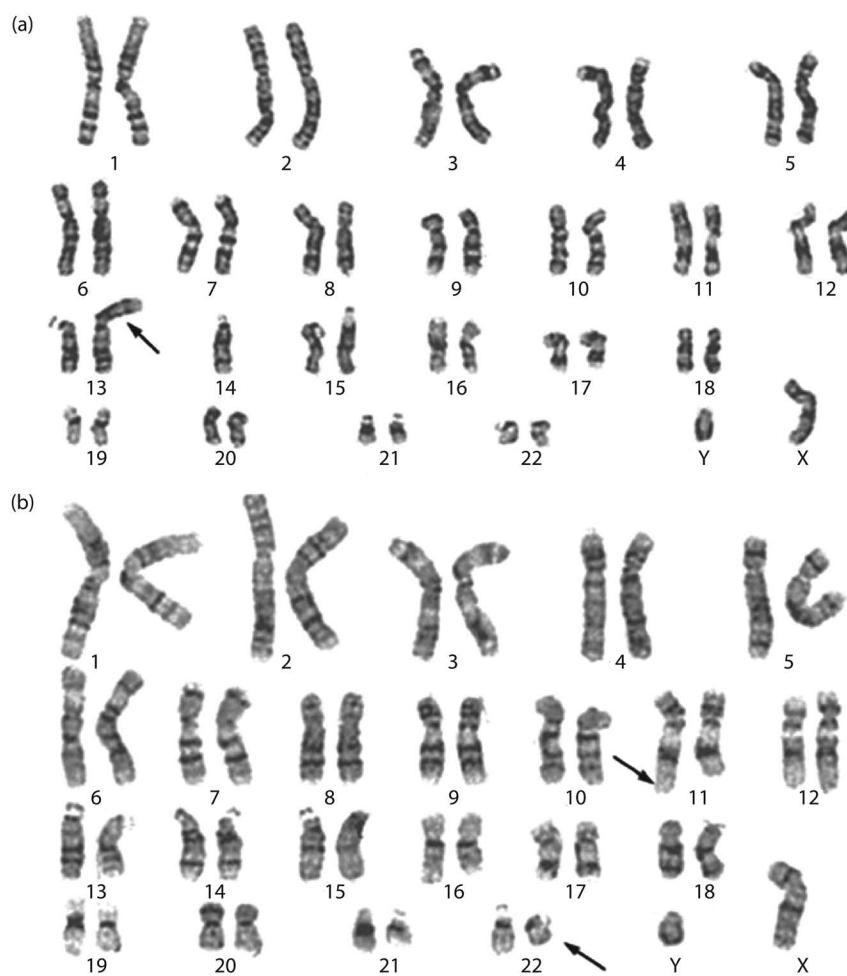


FIGURE 24.1 (a) 45,XY,der(13;14)(q10;q10) karyotype from a phenotypic normal male with a Robertsonian translocation of chromosomes 13 and 14 through centromeric fusion. (b) 46,XY,t(11;22)(q24.3;q12) karyotype from phenotypic normal male with a balanced reciprocal translocation of chromosome 11 and 22 with break points in 11q24.3 (↖) and 22q12 (↗).

deletions occur by intra-chromosomal homologous recombination between repeat sequences spread over the Yq11 region [17–19]. These repeat sequences are either palindromes consisting of inverted repeat arms or intra-chromosomal repetitive sequences. It is evident that if these microdeletions cause a spermatogenic defect leading to a low to very low sperm count present in the ejaculate or a few sperm cells in the testes, these microdeletions will, through the use of ICSI, be transmitted to sons, who most probably will be infertile as well [20]. In a few exceptional cases, fertility has been described in AZFc-deleted fathers who transmitted the deletion to their now-infertile sons [21–23]. Age at investigation may play a role, as observed in one

patient with an AZFc deletion being oligozoospermic and later on azoospermic [24].

CBAVD and cystic fibrosis

Men with CBAVD have obstructive azoospermia. Spermatogenesis is usually normal and sperm can be obtained through microsurgical epididymal sperm aspiration, testicular sperm extraction, percutaneous epididymal sperm aspiration, or epididymal or testicular fine-needle aspiration. This sperm can be used to fertilize oocytes *in vitro* through ICSI [2, 25]. CBAVD is known to be present in 97%–99% of male cystic fibrosis (CF) patients. CF is a frequent and by now well-known autosomal

TABLE 24.1 Incidence of Chromosomal Aberrations in Infertile Oligozoospermic and Azoospermic Males Compared with New-borns

Aberrations	Infertile Males (n = 7876)	Oligozoospermia (n = 1701)	Azoospermia (n = 1151)	New-borns (n = 94,465)
Autosomes	1.3%	3.0%	1.1%	0.25%
Sex chromosomes	3.8%	1.6%	12.6%	0.14%
Total	5.1%	4.6%	13.7%	0.39%

Source: Summarized from Van Assche E, Bonduelle M, Tournaye H et al. Cytogenetics of infertile men. *Hum Reprod* 1996; 11: 1–26.

recessive disease in the Caucasian population with an incidence of approximately 1/2500. Many patients, now surviving into their 30s and 40s, suffer from severe lung disease and pancreatic insufficiency. Although they are often too ill to reproduce, improved survival into adulthood generates interest in reproduction [26, 27]. The *CFTR* gene, encoding a protein involved in chloride transport across epithelial membranes, was shown to be responsible for CF due to malfunction of the protein when mutated [28–30].

CBAVD had also been observed in 1%–2% of apparently healthy infertile males, and in 6%–10% of men with obstructive azoospermia [31]. When the *CFTR* gene was studied in these males, pathogenic variants or splice-site variants in intron 8 (comprising the so-called 5T variant and the TG dinucleotide repeat upstream of it) interfering with gene expression were found in 80%–90% of them [32–37]. In the remaining CBAVD patients, no link could be found with aberrant *CFTR* expression. However, in these patients, CBAVD-associated urinary tract/renal malformations were observed [34, 38]. In a small group of patients with CBAVD, pathogenic variants are present in the *ADGRG2* gene [39]. When performing ICSI with sperm from CBAVD males carrying *CFTR* mutations, their partners have to be tested as well since the carrier frequency of pathogenic variants in the *CFTR* gene may be as high as 1/25 in Caucasians. If both partners carry pathogenic *CFTR* variants, the risk of having a child with CF is as high as ¼, or 25%, or even ½, or 50% (Table 24.2). However, since the incidence and the type of *CFTR* mutations vary with ethnic origin as well as with geographical region, counselling and approaches to treatment will have to be adjusted. In high-risk situations, prenatal diagnosis or pre-implantation genetic diagnosis (PGD) is indicated (see later).

Genetic causes of globozoospermia and macrozoospermia

Globozoospermia is a rare (<0.1%) cause of male infertility. A major characteristic of these round-headed spermatozoa is the malformation or absence of the acrosome [40, 41]. The best-studied genes associated with this form of teratozoospermia in humans are *SPATA16*, *PICK1*, and *DPY19L2* [42–44]. In all of these cases, the condition is inherited as an autosomal recessive

disease. Variants in the *DPY19L2* gene are the most prevalent and can be detected in 60%–83.3% of patients with (type I) globozoospermia. Around 26.7%–73.3% of these patients are homozygous for a 200 kb deletion of the *DPY19L2* gene [45]. Pathogenic variants in *PICK1* and *SPATA16* have also been observed in patients with globozoospermia, although the prevalence is very low. The number of genes (potentially) involved in globozoospermia is increasing. However, also for these genes, the frequency of pathogenic variants is extremely low.

In another form of morphological abnormal spermatozoa (large-headed, multi-flagellar, polyploid spermatozoa), a condition resulting in male infertility is caused by pathogenic variants in the *AURKC* gene, which is involved in chromosomal segregation and cytokinesis [46]. The first alteration detected in this gene was a deletion of a single base pair (c.144delC). This pathogenic variant has been detected in patients of North African origin. Especially in a Magrebian population, it was estimated that ~1/50 are heterozygous for this variant. A second recurrent pathogenic variant (p.Tyr248*) can be detected in European patients [47].

Male infertility as part of a syndrome

Patients with infertility as part of a syndrome all have a 46,XY normal karyotype. Most of the defects are monogenic and for the majority of the disorders, either the underlying gene defect is known or a chromosomal locus is known or suggested [48]. Nevertheless, part of the male infertility syndromes remains idiopathic at the molecular level, and probably multiple genes are involved.

Myotonic dystrophy is a rather common autosomal dominant syndrome causing muscular dystrophy with an incidence of 1/8000. The presence of an expanded CTG trinucleotide repeat in the *DMPK* gene interferes with its function [49–53]. Symptoms can be very mild and restricted to cataract at an advanced age or, by contrast, very severe, as is the case in the congenital, often lethal form of the disease. Severity is related to the number of CTG repeats [44]. In 60%–80% of male patients, testicular tubular atrophy will develop resulting in OAT. When such spermatozoa are used to fertilize oocytes, the risk of transmitting the disease is ½, or 50%, often in a more severe form due

TABLE 24.2 Risk Calculations for a Child with Cystic Fibrosis (CF) or Congenital Bilateral Absence of the Vas Deferens (CBAVD) in a patient with CBAVD

	Male		Female		Risk
	8/10	×	1/25	×	
No testing	8/10	×	1/25	×	1/125
Testing female					
Carrier	8/10	×	1	×	1/4
No carrier	8/10	×	1/150	×	1/4
Testing male + female					
Female carrier	CF/CF	×	1	×	1/2
Female no carrier	CF/CF	×	1/150	×	1/2
Female carrier	CF/5T	×	1	×	1/4 (CF)
					=1/8 (CBAVD)

Note: If the CBAVD patient is not tested for the presence of pathogenic variants in the *CFTR* gene, his risk of having at least one pathogenic CF variant is 8/10; if his partner is not tested and Caucasian, her risk of being a carrier of one pathogenic *CFTR* variant is 1/25. A carrier or heterozygous individual has a risk of 1/2 to transmit the variant. Two carriers have a risk of 1/4 to transmit their mutated gene at the same time. A CBAVD patient with two pathogenic variants will always transmit a mutated gene. Risks for CF can be calculated if none of the partners is tested, if only the female partner is tested, and if both partners are tested. In high-risk situations, pre-conceptional or pre-implantation genetic diagnosis can be offered [91].

to further expansion of the trinucleotide repeat (called anticipation). Prenatal diagnosis or preferential pre-implantation testing should be offered [54, 55].

Hypogonadotropic hypogonadism, characterized by an impaired gonadotropin-releasing hormone secretion, is divided into two major groups based on the underlying pathogenic mechanism. If associated with anosmia, the term Kallmann syndrome is used. X-linked along with autosomal recessive and autosomal dominant inheritance forms exist. The X-linked form of Kallmann syndrome (caused by pathogenic variants in the *KAL1* gene) is the most frequent and the best-known one [56], see Figure 24.2b. An autosomal dominant form of Kallmann syndrome is caused by pathogenic variants in the *FGFR1* gene [57]. A possible interaction between the gene products of the *KAL1* and *FGFR1* genes

has been suggested as an explanation for the higher prevalence of Kallmann syndrome in males than in females [58, 59]. However, more than 30 genes involved in hypogonadotropic hypogonadism (including Kallmann syndrome) have been identified and this number is still increasing [60]. Nevertheless, in the majority of patients with a clinical diagnosis of Kallmann syndrome or idiopathic hypogonadotropic hypogonadism, no pathogenic variants can be detected.

Primary ciliary dyskinesia or immotile cilia syndrome is an autosomal recessive disease presenting with chronic respiratory tract disease, rhinitis, and sinusitis due to immotile cilia. Male patients are usually infertile because of asthenozoospermia [61]. If the aforementioned symptoms are associated with situs inversus, the condition is called Kartagener syndrome [62, 63].

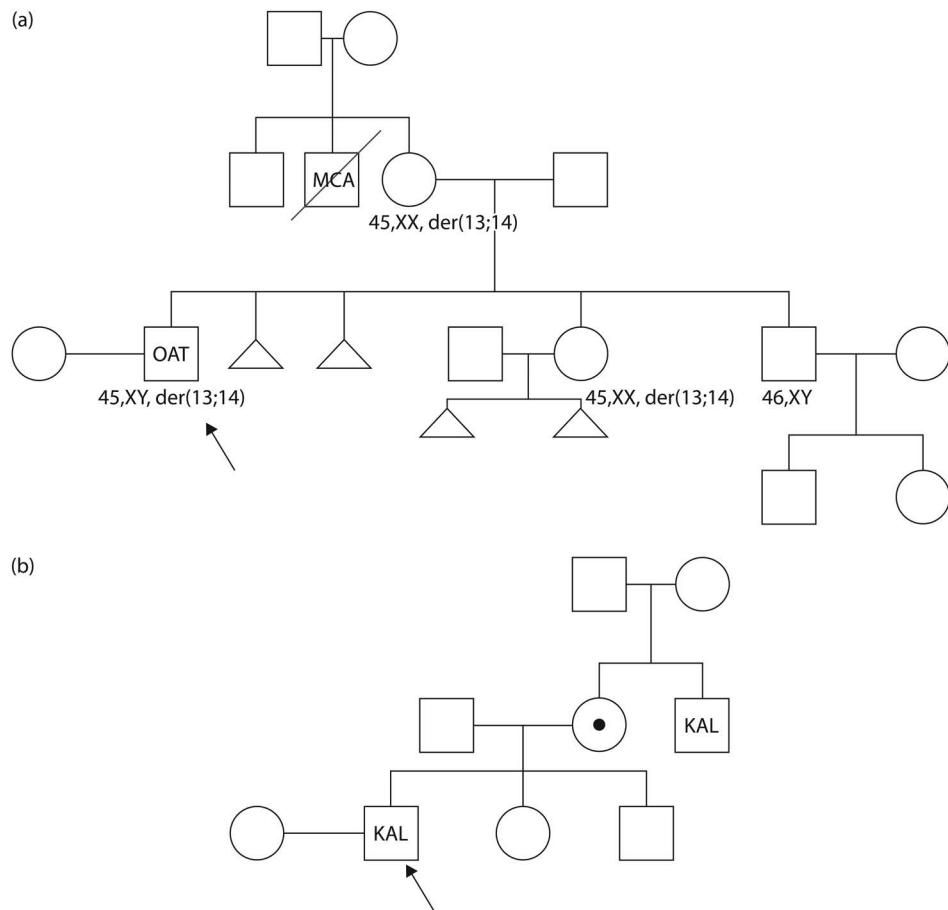


FIGURE 24.2 (a) Segregation of a Robertsonian translocation der(13;14) in a family. “OAT” (our proband ↳) presents with infertility due to oligo-astheno-teratozoospermia. His sister had two miscarriages (Δ); his brother has two healthy children. His mother had two miscarriages (Δ), lost a brother born with multiple congenital anomalies (MCA), and has a healthy brother without children. This story is suggestive of a chromosomal translocation. The karyotype of “OAT” points to a Robertson translocation der(13;14) (Figure 24.1a). His mother and his sister have the same translocation explaining the recurrent miscarriages (Δ). These miscarriages are most probably resulting from a trisomy 14 or monosomy 13 or 14. The brother of “OAT” has a normal karyotype, which is perfectly possible. The MCA brother of the mother died and most probably had trisomy 13. “OAT” should be informed about all these possible risks in case of pregnancy. In case of intracytoplasmic sperm injection, a pre-implantation genetic diagnosis or a prenatal diagnosis should be offered. (b) X-linked Kallmann syndrome in a family: its consequences and recommendations. “KAL” (our proband ↳) has Kallmann syndrome. The family history fits with an X-linked transmission since the brother of the mother of “KAL” has the same disease. This means that the mother of “KAL” must be a carrier ⚭. Her daughter, the sister of our proband, therefore has a 50% risk of being a carrier and a 25% risk of having an affected son. Pre-implantation or prenatal diagnosis should be discussed. If the wife of “KAL” becomes pregnant, boys will be healthy and fertile (because they inherit the Y chromosome of their father), while girls will always be carriers.

Men with this condition can reproduce with the help of ICSI. Unfortunately, genetic counselling is hampered because of the lack of knowledge of all genes involved in primary ciliary dyskinesia and Kartagener syndrome [63, 64].

Kennedy's disease, or spinal and bulbar muscular atrophy, is a neuromuscular disease that causes muscular weakness and is associated with testicular atrophy which leads to oligozoospermia or azoospermia. It is an X-linked disease caused by an expanded (CAG) trinucleotide repeat in the transactivation domain of the androgen receptor gene [65, 66]. If treated with ICSI, genetic counselling is again indicated. However, point mutations in the androgen receptor gene might result in androgen insensitivity through impaired binding of dihydrotestosterone to the receptor, which will interfere with sexual development. The resulting syndrome is testicular feminization or androgen insensitivity syndrome, causing a (partial) female phenotype [67, 68]. The presenting problem here will not (only) be male infertility. Similarly, patients with an autosomal recessive 5α-reductase deficiency and therefore unable to synthesize dihydrotestosterone from testosterone may theoretically present at the clinic with azoospermia and pseudo-hermaphroditism [69, 70].

Very rarely, patients with other (mostly) syndrome-associated genetic defects may consult at a male infertility clinic. Up to 80% of patients with Noonan syndrome present with oligozoospermia or azoospermia as a result of cryptorchidism [71]. The diagnosis is often based on other symptoms, including small stature, chest deformity, a rather typical facial dysmorphism, and congenital heart disease. Defects in the *PTPN11* gene are responsible for approximately 40% of patients with Noonan syndrome [72]. Other genes involved in Noonan syndrome have been identified and, altogether, around 60% of cases can be solved. Consequently, more (currently unknown) genes must be involved in Noonan syndrome. The autosomal dominant inheritance pattern asks for genetic counselling. Other possible patients may be affected by Aarskog–Scott syndrome with acrosomal sperm defects [73, 74] or Beckwith–Wiedemann syndrome with cryptorchidism [75]. Syndromes such as Bardet–Biedl syndrome and Prader–Willi syndrome, both presenting with hypogonadism, are associated with other major symptoms, including (severe) mental retardation, which limit procreation [76, 77]. Prader–Willi syndrome is an imprinting syndrome resulting from the absence of expression of the paternal alleles in the 15q11–q13 imprinted region [78–80]. Other causes of male infertility include deficiencies in enzymes involved in the synthesis of testosterone, luteinizing hormone, and luteinizing hormone receptor [81, 82].

Also, defects in energy production by the mitochondria have been implicated in male infertility. Mitochondria are the main sources of energy production for the cells through the process of oxidative phosphorylation. The synthesis of ATP occurs through the action of five enzyme complexes that are encoded by both nuclear genes and the small mitochondrial genome that is exclusively maternally inherited. Mitochondrial diseases usually evolve as multisystem disorders mainly affecting the central nervous system and muscles. In addition, these defects in respiratory function are believed to cause a decline in sperm motility because of the depletion of ATP, which is necessary for the flagellar propulsion of the spermatozoa. Reduced sperm motility and resulting male infertility have been well documented in several patients with mitochondrial encephalopathies caused by mitochondrial tRNA point mutations or (multiple) mtDNA deletions [83].

Genome-wide testing strategies

Since the number of genes known to be causal for male infertility is increasing, there is a great need for genome-wide analyses. For the detection of copy number variations (deletions/duplications), the diagnostic yield and utility of array comparative genomic hybridization has been investigated in multiple studies. Mostly, these results were disappointing. Besides for deletions involving the *MAGEA9* gene, the detected copy number variations were either of unknown clinical significance or not confirmed in subsequent studies [84, 85]. It might, however, be worthwhile to look for copy number variations in regions known to be related to male infertility or containing genes involved in male infertility.

Gene panel analyses have been proven to be successful for conditions/syndromes involving multiple genes, such as hypogonadotropic hypogonadism. Furthermore, gene panel analyses as well as exome sequencing have been applied in view of idiopathic male infertility where azoospermia or severe OAT is the sole symptom [86, 87]. At this moment, however, there is no consensus about the genes to be included in such a gene panel. Again, only a small part of the suggested infertility genes or variants could be confirmed in subsequent studies [88]. When looking at recent literature, it is evident that the number of genes potentially involved in idiopathic male infertility is still increasing, and most likely genes still need to be discovered. In order to avoid updating panels on a regular basis, one might opt to perform exome sequencing. Nevertheless, the more genes that are analysed simultaneously, the more complex the interpretation is.

Consequences and recommendations in the clinic

Genetic evaluation of infertile males before ART use

A personal history from the patient should be taken. In addition, a detailed pedigree should be drawn and completed for miscarriages or children (also deceased) with multiple congenital malformations in first- or second-degree relatives. It is also important to know about the infertility status of siblings or other family members. This information may suggest a possible chromosomal aberration such as a translocation or a monogenic disease like Kallmann syndrome or CF. A thorough inquiry of the proband and his partner may pinpoint other hereditary diseases not necessarily causing infertility but causing morbidity or being lethal to offspring. A complete clinical examination of the proband and his partner is useful for establishing a clinical diagnosis of a disease or a syndrome associated with infertility such as Klinefelter syndrome or CF-linked CBAVD. This examination may also reveal other possible hereditary diseases not identified before. Since the couple is in such a case not aware of a genetic problem, they should be counselled before starting the treatment. Complementary tests—mainly laboratory investigations—will help to confirm a clinical diagnosis. In case of male infertility, the personal history, the clinical examination, a semen analysis, and hormonal tests are sufficient to characterize most of the patients as being:

1. Infertile in association with other physical or mental problems.
2. Infertile but otherwise healthy. These patients can mostly be subdivided into oligozoospermic or eventually OAT males and into males with obstructive or non-obstructive azoospermia. Rarely, patients with teratozoospermia are detected through semen analysis.

Genetic investigations will help to refine the diagnosis and to counsel the patient/couple accordingly. The preceding information will help to select additional tests to be performed. In most cases of male infertility due to severe OAT or non-obstructive azoospermia, a peripheral karyotype should be performed, even if the family history is not suggestive of a chromosomal disorder [6–8]. In the same cohort of patients, microdeletions of the AZF regions on Yq11 should be looked for in DNA from peripheral blood. The possibility of fertility treatment in couples in whom the male has an AZF deletion is strongly dependent on the type of deletion present [89]. Deletions of AZFa or AZFb, or combinations including these regions, have a bad prognosis since no sperm cells will be produced and ICSI will not be possible. In contrast, spermatozoa can be found in about 70% of patients with a complete deletion of the AZFc region [89]. For these patients ICSI will be possible.

In men with non-obstructive azoospermia caused by CBAVD without anomalies of the urogenital tract, pathogenic variants in the *CFTR* gene should be looked for in the patient and, even more importantly, in his partner. At present it is possible to identify 85%–90% of heterozygous individuals (“carriers”) in the Caucasian population [90, 91]. Depending on whether *CFTR* mutations have been identified in the male patient and/or his female partner, the risk of conceiving a child with CF can be calculated (Table 24.2). These figures together with the type of alterations may be an indication for prenatal diagnosis or preimplantation genetic testing (PGT)13 [26, 92, 93]. More specific tests should be performed if diseases such as Kennedy disease, Kallmann syndrome, myotonic dystrophy, immotile cilia syndrome, or other syndromes or diseases are suspected. In these cases, it is again important not only to establish a correct diagnosis to treat appropriately, but also to counsel the proband and his family adequately concerning recurrence risks and prenatal diagnosis (PND) or preimplantation genetic testing (PGT).

Genetic testing during ART use for severe male infertility

Genetic tests that can be performed during ART refer to PGT. They involve the genetic testing (PGT) of embryos before implantation [94–99]. The aim is to avoid the birth of a child with a genetic disease. PGT makes conventional prenatal diagnosis, eventually followed by termination of pregnancy, obsolete. PGT is a complex procedure because of the “single-cell” genetic diagnosis. It was developed and first applied in the clinic more than 30 years ago [98]. At first, most of the PGTs performed were for CF, myotonic dystrophy, Huntington’s disease, and Duchenne muscular dystrophy, but many others have since been performed for either infertile or fertile couples [96, 97]. For chromosomal aberrations, most PGTs have been done for reciprocal and Robertsonian translocations [94, 99]. An example of a family with a Robertsonian translocation der(13;14) is visualized in Figure 24.2a. In general, the take-home baby rate is of the same order of magnitude of 20%–25% as in ICSI cycles in general [2, 3]. A number of PGTs have been performed for Klinefelter patients in whom spermatozoa found in the testes were used to fertilize oocytes [13].

Genetic evaluation of pregnancies and children conceived through ICSI because of severe male infertility

Follow-up studies of pregnancies established and children born after the use of ICSI have been initiated as soon as this new procedure was applied in the clinic. From these still ongoing studies, it

became clear that the number of major malformations was comparable to the number of major malformations in *in vitro* fertilization (IVF) children, and possibly slightly higher than in naturally conceived children. The results of the psychomotor development of these children are also reassuring [100–107]. Current investigations are looking at the reproductive profiles of young adults born after ICSI [108–111].

The *de novo* chromosomal aberrations found at prenatal diagnosis indicate that numerical sex chromosomal anomalies are slightly increased when compared to a large newborn population. The incidence in the newborn after natural conception is 0.2%, but the incidence in ICSI children is 0.8%. This is a fourfold increase, but of course the overall incidence remains low (<1%). Apart from sex chromosome anomalies, *de novo*-balanced translocations have also been observed [105, 107, 112]. These aberrations occurring in children of men with a normal peripheral karyotype could be related to chromosomal anomalies being present in their sperm but not in their lymphocytes [113–116].

Conclusion

In case of severe male infertility, good clinical practice requires genetic evaluation before, during, and after ART in order to properly treat and counsel the proband, the couple, and, eventually, the family. The aim is to inform the patients about possible risks, to improve the success rate of the ART treatment, and to avoid the birth of children affected with a severe genetic disease. Moreover, at present there are still many unknown causes of male infertility. More research in the field of genetics will provide us with a better understanding, along with a better defining of how great the risks are of transmitting infertility or possibly other genetic anomalies to the next generation.

References

1. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340:17–8.
2. Devroey P, Van Steirteghem A. A review of ten years experience of ICSI. Hum Reprod Update. 2004;10:19–28.
3. The ESHRE Capri Workshop Group. Intracytoplasmic sperm injection (ICSI) in 2006: Evidence and evolution. Hum Reprod Update. 2007;13:515–26.
4. Jacobs PA, Strong JA. A case of human intersexuality having a possible XXY sex-determining mechanism. Nature. 1959;183:302–3.
5. Forti G, Corona G, Vignozzi L, et al. Klinefelter’s syndrome: A clinical and therapeutical update. Sex Dev. 2010;4:167–9.
6. Van Assche E, Bonduelle M, Tournaye H, et al. Cytogenetics of infertile men. Hum Reprod. 1996;11:1–26.
7. Yoshida A, Miura K, Shirai M, et al. Cytogenetic survey of 1007 infertile males. Urol Int. 1997;58:166–76.
8. Martin RH. Cytogenetic determinants of male fertility. Hum Reprod Update. 2008;14:379–90.
9. Weil D, Wang I, Dietrich A, et al. Highly homologous loci on the X and Y chromosomes are hot-spots for ectopic recombination resulting in XX maleness. Nat Genet. 1994;7:414–9.
10. Schiebel K, Winkelmann M, Mertz A, et al. Abnormal XY interchange between a novel isolated protein kinase gene, *PRKY*, and its homologue, *PRKX*, accounts for one third of all (Y+) XX males and (Y-) XY females. Hum Mol Genet. 1997;6:1985–9.
11. Vorona E, Zitzmann M, Gromoll J, et al. Clinical, endocrinological, and epigenetic features of the 46, XX male syndrome, compared with 47,XXY Klinefelter patients. J Clin Endocrinol Metab. 2007;92:3458–65.

12. Sinclair AH, Berta P, Palmer MS, et al. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature*. 1990;346:240–4.
13. Fullerton G, Hamilton M, Maheshwari A. Should non-mosaic Klinefelter syndrome men be labelled as infertile in 2009? *Hum Reprod*. 2010;25:588–97.
14. Tiepolo L, Zuffardi O. Localization of factors controlling spermatogenesis in the nonfluorescent position of the human Y chromosome long arm. *Hum Genet*. 1976;34:119–24.
15. Krausz C, Hoefsloot L, Simoni M, Tüttelmann F. European Academy of andrology; European Molecular genetics quality network. EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: State-of-the-art 2013. *Andrology*. 2014;2:5–19.
16. Massart A, Lissens W, Tournaye H, Stouffs K. Genetic causes of spermatogenic failure. *Asian J Androl*. 2012;14:40–8.
17. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, et al. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature*. 2003;423:825–37.
18. Repping S, Skaletsky H, Lange J, et al. Recombination between palindromes P5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. *Am J Hum Genet*. 2002;71:906–22.
19. Jobling MA. Copy number variation on the human Y chromosome. *Cytogenet Genome Res*. 2008;123:253–62.
20. Silber SH. The Y chromosome in the era of intracytoplasmic sperm injection: A personal view. *Fertil Steril*. 2011;95:2439–48.
21. Saut N, Terriou P, Navarro A, et al. The human Y chromosome genes *BPY2*, *CDY1* and *DAZ* are not essential for sustained fertility. *Mol Hum Reprod*. 2000;6:789–93.
22. Chang PL, Sauer MV, Brown S, et al. Y chromosome microdeletion in a father and his four infertile sons. *Hum Reprod*. 1999;14:2689–94.
23. Calogero AE, Garofalo MR, Barone N, et al. Spontaneous transmission from a father to his son of a Y chromosome microdeletion involving the deleted in azoospermia (*DAZ*) gene. *J Endocrinol Invest*. 2002;25:631–4.
24. Girardi SK, Mielnik A, Schlegel PN. Submicroscopic deletions in the Y chromosome of infertile men. *Hum Reprod*. 1997;12:1635–41.
25. Sarkar NN. Intracytoplasmic sperm injection: An assisted reproductive technique and its outcome to overcome infertility. *J Obstet Gynaecol*. 2007;27:347–53.
26. Sueblinvong V, Whittaker LA. Fertility and pregnancy: Common concerns of the aging cystic fibrosis population. *Clin Chest Med*. 2007;28:433–43.
27. Keymolen K, Goossens V, De Rycke M, et al. Clinical outcome of preimplantation genetic diagnosis for cystic fibrosis: The Brussels' experience. *Eur J Hum Genet*. 2007;15:752–8.
28. Kerem B, Rommens JM, Buchanan JA, et al. Identification of the cystic fibrosis gene: Genetic analysis. *Science*. 1989;245:1073–80.
29. Riordan JR, Rommens JM, Kerem B, et al. Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science*. 1989;245:1066–73.
30. Rommens JM, Iannuzzi MC, Kerem B, et al. Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science*. 1989;245:1059–65.
31. Dubin L, Amelar RD. Etiologic factors in 1294 consecutive cases of male infertility. *Fertil Steril*. 1971;22:469–74.
32. Anguiano A, Oates RD, Amos JA, et al. Congenital bilateral absence of the vas deferens. A primarily genital form of cystic fibrosis. *JAMA*. 1992;267:1794–7.
33. Chillon M, Casals T, Mercier B, et al. Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens. *N Engl J Med*. 1995;332:1475–80.
34. Clastres M. Molecular pathology of the *CFTR* locus in male infertility. *Reprod Biomed Online*. 2005;10:14–41.
35. Cuppens H, Cassiman JJ. *CFTR* mutations and polymorphisms in male infertility. *Int J Androl*. 2004;27:251–6.
36. Lissens W, Mercier B, Tournaye H, et al. Cystic fibrosis and infertility caused by congenital bilateral absence of the vas deferens and related clinical entities. *Hum Reprod*. 1996;11(Suppl 4): 55–80.
37. Groman JD, Hefferon TW, Casals T, et al. Variation in a repeat sequence determines whether a common variant of the cystic fibrosis transmembrane conductance regulator gene is pathogenic or benign. *Am J Hum Genet*. 2004;74:176–9.
38. Dumur V, Gervais R, Rigot JM, et al. Congenital bilateral absence of the vas deferens in absence of cystic fibrosis. *Lancet*. 1995;345:200–1.
39. Patat O, Pagan A, Siegfried A, et al. Truncating Mutations in the Adhesion G Protein-Coupled Receptor G2 Gene ADGRG2 Cause an X-Linked Congenital Bilateral Absence of Vas Deferens. *Am J Hum Genet*. 2016;99(2):437–42.
40. Dam AH, Feenstra I, Westphal JR, et al. Globozoospermia revisited. *Hum Reprod Update*. 2007;13:63–75.
41. Coutton C, Escouffier J, Martinez G, et al. Teratozoospermia: Spotlight on the main genetic actors in the human. *Hum Reprod Update*. 2015;21:455–85.
42. Dam AH, Koscienski I, Kremer JA, et al. Homozygous mutation in *SPATA16* is associated with male infertility in human globozoospermia. *Am J Hum Genet*. 2007;81: 813–20.
43. Liu G, Shi QW, Lu GX. A newly discovered mutation in *PICK1* in a human with globozoospermia. *Asian J Androl*. 2010;12:556–60.
44. Harbuz R, Zouari R, Pierre V, et al. Recurrent deletion of *DPY19L2* causes infertility in man by blocking sperm head elongation and acrosome formation. *Am J Hum Genet*. 2011;88:351–61.
45. Koscienski I, Ellnati E, Fossard C, et al. *DPY19L2* deletion as a major cause of globozoospermia. *Am J Hum Genet*. 2011;88:344–50.
46. Dieterich K, Soto Rifo R, Faure AK, et al. Homozygous mutation of *AURKC* yields large-headed polyploid spermatozoa and causes male infertility. *Nat Genet*. 2007;39:661–5.
47. Ben Khelifa M, Zouari R, Harbuz R, et al. Identification of a new recurrent aurora kinase c mutation in both European and African men with macrozoospermia. *Mol Hum Reprod*. 2011;17:762–8.
48. Lissens W, Liebaers I, Van Steirteghem A. Male infertility. In: *Emery and Rimoin's Principles and Practice of Medical Genetics*. Rimoin DL, Connor JM, Pyeritz RE, Korf BC (eds.). Philadelphia, PA: Elsevier, pp. 856–74, 2007.
49. Aslanidis C, Jansen G, Amemiya C, et al. Cloning of the essential myotonic dystrophy region and mapping of the putative defect. *Nature*. 1992;355:548–51.
50. Brook DJ, McCurrach ME, Harley HG, et al. Molecular basis of myotonic dystrophy: Expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell*. 1992;68:799–808.
51. Fu HY, Pizzuti A, Fenwick RG, et al. An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science*. 1992;255:1256–8.
52. Mahadevan M, Tsilfidis C, Sabourin L, et al. Myotonic dystrophy mutation: An unstable CTG repeat in the 3' untranslated region of the gene. *Science*. 1992;255:1253–5.
53. Hunter A, Tsilfidis C, Mettler G, et al. The correlation of age of onset with CTG trinucleotide repeat amplification in myotonic dystrophy. *J Med Genet*. 1992;29:774–9.
54. Sermon K, De Vos A, Van de Velde H, et al. Fluorescent PCR and automated fragment analysis for the clinical application of pre-implantation genetic diagnosis of myotonic dystrophy (Steinert's disease). *Mol Hum Reprod*. 1998;4:791–6.
55. Sermon K, Seneca S, De Rycke M, et al. PGD in the lab for triplet repeat diseases—Myotonic dystrophy, Huntington's disease and fragile-X syndrome. *Mol Cell Endocrinol*. 2001; 183(Suppl 1): S77–85.
56. Rugarli EI, Ballabio A. Kallmann syndrome. From genetics to neurobiology. *JAMA*. 1993;270:2713–16.
57. Dodé C, Levilliers J, Dupont JM, et al. Loss-of-function mutations in *FGFR1* cause autosomal dominant Kallmann syndrome. *Nat Genet*. 2003;33:463–5.

58. Ayari B, Soussi-Yanicostas N. *FGFR1* and anosmin-1 underlying genetically distinct forms of Kallmann syndrome are co-expressed and interact in olfactory bulbs. *Dev Genes Evol.* 2007;217:169–75.
59. Cadman SM, Kim SH, Hu Y, et al. Molecular pathogenesis of Kallmann's syndrome. *Horm Res.* 2007;67:231–42.
60. Louden ED, Poch A, Kim HG, Ben-Mahmoud A, Kim SH, Layman LC. Genetics of hypogonadotropic hypogonadism-human and mouse genes, inheritance, oligogenicity, and genetic counseling. *Mol Cell Endocrinol.* 2021;534:111334.
61. Cardenas-Rodriguez M, Badano JL. Ciliary biology: Understanding the cellular and genetic basis of human ciliopathies. *Am J Med Genet.* 2009;151:263–80.
62. Afzelius BA. Immotile cilia syndrome: Past, present, and prospects for the future. *Thorax.* 1998;53:894–7.
63. Sutherland MJ, Ware SM. Disorders of left-right asymmetry: Heterotaxy and situs inversus. *Am J Med Genet.* 2009;151:307–17.
64. Baker K, Beales PL. Making sense of cilia in disease: The human ciliopathies. *Am J Med Genet.* 2009;151C:281–95.
65. Igarashi S, Tanno Y, Onodera O, et al. Strong correlation between the number of CAG repeats in androgen receptor genes and the clinical onset features of spinal and bulbar atrophy. *Neurology.* 1992;42:2300–2.
66. Finsterer J. Perspectives of Kennedy's disease. *J Neurol Sci.* 2010;298:1–10.
67. Quigley CA, De Bellis A, Marschke KB, et al. Androgen receptor defects: Historical, clinical and molecular perspectives. *Endocr Rev.* 1995;16:271–321.
68. Wisniewski AB, Mazur T. 46,XY DSD with female or ambiguous external genitalia at birth due to androgen insensitivity syndrome, 5α-reductase-2 deficiency, or 17-hydroxysteroid dehydrogenase deficiency: A review of quality of life outcomes. *Int J Pediatr Endocrinol.* 2009;2009:567430.
69. Sinnecker GH, Hiort O, Dibbelt L, et al. Phenotypic classification of male pseudo hermaphroditism due to steroid 5α-reductase 2 deficiency. *Am J Med Genet.* 1996;63:223–30.
70. Chong CK. Practical approach to steroid 5alphareductase type 2 deficiency. *Eur J Pediatr.* 2011;170:1–8.
71. Romano A, Allanson J, Dahlgren J, et al. Noonan syndrome: Clinical features, diagnosis and management guidelines. *Pediatrics.* 2010;126:746–59.
72. Tartaglia M, Mehler EL, Goldberg R, et al. Mutations in *PTPN11*, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. *Nat Genet.* 2001;29:465–8.
73. Meschede D, Rolf C, Neugebauer DC, et al. Sperm acrosome defects in a patient with Aarskog–Scott syndrome. *Am J Med Genet.* 1996;66:340–2.
74. Orrico A, Galli L, Faivre L, et al. Aarskog–Scott syndrome: Clinical update and report of nine novel mutations in the *FGD1* gene. *Am J Med Genet.* 2010;152:313–18.
75. Choufani S, Shuman C, Weksberg R. Beckwith–Wiedemann syndrome. *Am J Med Genet.* 2010;154C:343–54.
76. Beales PL, Elcioglu N, Woolf AS, et al. New criteria for improved diagnosis of Bardet–Biedl syndrome: Results of a population survey. *J Med Genet.* 1999;36:437–46.
77. Cassidy SB. Prader–Willi syndrome. *J Med Genet.* 1997;34:917–23.
78. Horsthemke B, Dittrich B, Buiting K, et al. Imprinting mutations on human chromosome 15. *Hum Mutat.* 1997;10:329–37.
79. Feil R, Khosla S. Genomic imprinting in mammals. *Trends Genet.* 1999;15:431–5.
80. Vogels A, Moerman P, Frijns JP, Bogaert GA. Testicular histology in boys with Prader–Willi syndrome: Fertile or infertile? *J Urol.* 2008;180:1800–4.
81. Weiss J, Axelrod L, Whitcomb RW, et al. Hypogonadism caused by a single amino acid substitution in the beta subunit of luteinizing hormone. *N Engl J Med.* 1992;326:179–83.
82. Latronico AC, Segaloff DL. Naturally occurring mutations of the luteinizing-hormone receptor: Lessons learned about reproductive physiology and G protein-coupled receptors. *Am J Hum Genet.* 1999;65:949–58.
83. Rajender S, Rahul P, Mahdi AA. Mitochondria, spermatogenesis and male infertility. *Mitochondrion.* 2010;10:419–28.
84. Lo Giacco D, Chianese C, Ars E, Ruiz-Castañé E, Forti G, Krausz C. Recurrent X chromosome-linked deletions: Discovery of new genetic factors in male infertility. *J Med Genet.* 2014;51(5):340–344.
85. Stouffs K, Vandermaelen D, Massart A, Menten B, Vergult S, Tournaye H, Lissens W. Array comparative genomic hybridization in male infertility. *Hum Reprod.* 2012;27(3):921–9.
86. Oud MS, Ramos L, O'Bryan MK, McLachlan RI, Okutman Ö, Viville S, de Vries PF, Smeets DFCM, Lugtenberg D, Hehir-Kwa JY, Gilissen C, van de Vorst M, Vissers LELM, Hoischen A, Meijerink AM, Fleischer K, Veltman JA, Noordam MJ. Validation and application of a novel integrated genetic screening method to a cohort of 1,112 men with idiopathic azoospermia or severe oligozoospermia. *Hum Mutat.* 2017;38(11):1592–1605.
87. Kherraf ZE, Cazin C, Bouker A, Fourati Ben Mustapha S, Hennebicq S, Septier A, Coutton C, Raymond L, Nouchy M, Thierry-Mieg N, Zouari R, Arnould C, Ray PF. Whole-exome sequencing improves the diagnosis and care of men with non-obstructive azoospermia. *Am J Hum Genet.* 2022;109(3):508–17.
88. Houston BJ, Riera-Escamilla A, Wyrwoll MJ, Salas-Huetos A, Xavier MJ, Nagirnaja L, Friedrich C, Conrad DF, Aston KI, Krausz C, Tüttelmann F, O'Bryan MK, Veltman JA, Oud MS. A systematic review of the validated monogenic causes of human male infertility: 2020 update and a discussion of emerging gene-disease relationships. *Hum Reprod Update.* 2021;28(1):15–29.
89. Stouffs K, Lissens W, Tournaye H, et al. The choice and outcome of the fertility treatment of 38 couples in whom the male partner has a Yq microdeletion. *Hum Reprod.* 2005;20:1887–96.
90. Dequeker E, Stuhrmann M, Morris MA, et al. Best practice guidelines for molecular genetic diagnosis of cystic fibrosis and CFTR-related disorders—Updated European recommendations. *Eur J Hum Genet.* 2009;17:51–65.
91. World Health Organisation. *The molecular genetic epidemiology of cystic fibrosis. Report of a joint meeting of WHO/ECFTN/ICF(M)A/ECFS,* 2004. Available from: www.who.int/genomics/publications/en/
92. Goossens V, Sermon K, Lissens W, et al. Clinical application of preimplantation genetic diagnosis for cystic fibrosis. *Prenat Diagn.* 2000;20:571–81.
93. Dreesen JC, Jacobs LJ, Bras M, et al. Multiplex PCR of polymorphic markers flanking the *CFTR* gene: A general approach for preimplantation genetic diagnosis of cystic fibrosis. *Mol Hum Reprod.* 2000;6:391–6.
94. van Montfoort A, Carvalho F, Coonen E, Kokkali G, Moutou C, Rubio C, Goossens V, De Rycke M. ESHRE PGT consortium data collection XIX-XX: PGT analyses from 2016 to 2017. *Hum Reprod.* Open. 2021;2021(3).
95. Sermon K, Van Steirteghem A, Liebaers I. Preimplantation genetic diagnosis. *Lancet.* 2004;363:1633–41.
96. Geraedts JP, De Wert GM. Preimplantation genetic diagnosis. *Clin Genet.* 2009;76:315–25.
97. Simpson JL. Preimplantation genetic diagnosis at 20 years. *Prenat Diagn.* 2010;30:682–95.
98. Handyside AH, Kontogianni EH, Hardy K, et al. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature.* 1990;344:768–70.
99. Harper JC, Coonen E, De Rycke M, et al. ESHRE PGD consortium data collection X: Cycles from January to December 2007 with pregnancy followup to October 2008. *Hum Reprod.* 2010;25:2685–707.
100. Bonduelle M, Liebaers I, Deketelaere V, et al. Neonatal data on a cohort of 2889 infants born after intracytoplasmic sperm injection (ICSI) (1991–1999) and of 2995 infants born after *in vitro* fertilization (IVF) (1983–1999). *Hum Reprod.* 2002;17:671–94.

101. Bonduelle M, Van Assche E, Joris H, et al. Prenatal testing in ICSI pregnancies: Incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. *Hum Reprod.* 2002;17:2600–14.
102. Bonduelle M, Ponjaert I, Van Steirteghem A, et al. Developmental outcome of children born after ICSI compared to children born after IVF at the age of two years. *Hum Reprod.* 2003;19:1–9.
103. Leunens L, Celestin-Westreich S, Bonduelle M, Liebaers I, Ponjaert-Kristoffersen I. Cognitive and motor development of 8-year-old children born after ICSI compared to spontaneously conceived children. *Hum Reprod.* 2006;21:2922–9.
104. Sutcliffe AG, Ludwig M. Outcome of assisted reproduction. *Lancet.* 2007;370:351–9.
105. Belva F, De Schrijver F, Tournaye H, et al. Neonatal outcome of 724 children born after ICSI using non-ejaculated sperm. *Hum Reprod.* 2011; 17:52–8.
106. De Schepper J, Belva F, Schietecatte J, et al. Testicular growth and tubular function in prepubertal boys conceived by intracytoplasmic sperm injection. *Horm Res.* 2009;71:359–63.
107. Woltringh GH, Besseling DE, Tillem AJ, et al. Karyotyping, congenital anomalies and follow-up of children after intracytoplasmic sperm injection with non-ejaculated sperm: A systemic review. *Hum Reprod Update.* 2010;16:12–19.
108. Belva F, Bonduelle M, Tournaye H. Endocrine and reproductive profile of boys and young adults conceived after ICSI. *Curr Opin Obstet Gynecol.* 2019;31(3):163–169.
109. Belva F, Roelants M, Vloeberghs V, Schietecatte J, Evenepoel J, Bonduelle M, de Vos M. Serum reproductive hormone levels and ultrasound findings in female offspring after intracytoplasmic sperm injection: First results. *Fertil Steril.* 2017;107(4):934–939.
110. Belva F, Roelants M, De Schepper J, Van Steirteghem A, Tournaye H, Bonduelle M. Reproductive hormones of ICSI-conceived young adult men: The first results. *Hum Reprod.* 2017;32(2): 439–446.
111. Belva F, Bonduelle M, Roelants M, Michielsen D, Van Steirteghem A, Verheyen G, Tournaye H. Semen quality of young adult ICSI offspring: The first results. *Hum Reprod.* 2016;31(12): 2811–2820.
112. Belva F, Bonduelle M, Buysse A, Van den Bogaert A, Hes F, Roelants M, Verheyen G, Tournaye H, Keymolen K. Chromosomal abnormalities after ICSI in relation to semen parameters: Results in 1114 fetuses and 1391 neonates from a single center. *Hum Reprod.* 2020;35(9):2149–2162.
113. Martin RH. Genetics of human sperm. *J Assist Reprod Genet.* 1998;15:240–455.
114. Aran B, Blanco J, Vidal F, et al. Screening for abnormalities of chromosomes x, y, and 18 and for diploidy in spermatozoa from infertile men participating in an *in vitro* fertilization-intracytoplasmic sperm injection program. *Fertil Steril.* 1999;72: 696–701.
115. Vegetti W, Van Assche E, Frias A, et al. Correlation between semen parameters and sperm aneuploidy rates investigated by fluorescence *in-situ* hybridization in infertile men. *Hum Reprod.* 2000;15:351–65.
116. Egozcue S, Blanco J, Vendrell JM, et al. Human male infertility: Chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion. *Hum Reprod Update.* 2000; 6:93–105.

PRE-IMPLANTATION GENETIC TESTING FOR ANEUPLOIDY TO IMPROVE CLINICAL OUTCOMES

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Chromosome aneuploidy is common in human gametes and pre-implantation embryos and is a major cause of pregnancy failure, miscarriage, and still births, with an incidence at birth of <0.3% [1, 2]. Most aneuploidies originate in the oocyte through errors in maternal meiosis and these increase exponentially in women in their late 30s and early 40s [3]. This is associated with a sharp increase in the incidence of miscarriage and a corresponding decline in live birth rates (LBRs) in these women following *in vitro* fertilization (IVF) [4]. In principle, therefore, there is no clinical or ethical justification for the transfer of aneuploid embryos following IVF treatment for infertility, where the aim is to help couples have a healthy baby. The challenge, however, is to identify euploid and aneuploid embryos accurately without compromising their treatment.

Pre-implantation genetic testing for aneuploidy (PGT-A)

Pre-implantation genetic testing for aneuploidy (PGT-A) originally involved polar body or cleavage-stage biopsy and single-cell analysis using multicolour fluorescent *in situ* hybridization (FISH) with a limited number of chromosome-specific probes, typically 7–9 chromosomes [5, 6] (Table 25.1). However, several randomized controlled trials (RCTs) failed to show a benefit and a large trial in the Netherlands showed a significant reduction in live births [7]. In the 15 years since then, there have been major advances in whole genome amplification and copy number analysis for all 24 chromosomes, which began with microarray comparative genomic hybridization (array CGH), progressed to next-generation sequencing (NGS)-based tests and, more recently, NGS-based targeted sequencing of single-nucleotide polymorphism (SNP) markers to improve accuracy [8, 9]. Over the same period, there have also been significant improvements in culture to the blastocyst stage and cryopreservation by vitrification [10, 11]. Combining the dual selection of blastocyst culture with trophectoderm biopsy and PGT-A, followed by vitrification and transfer of euploid blastocysts in later unstimulated cycles, there have been several reports of exceptionally high implantation rates (IRs) and live births per transfer, demonstrating that this approach is highly effective for embryo selection [12–14]. Furthermore, a recent non-selection study, in which blastocysts were selected based on morphology alone and biopsied before transfer but only tested after clinical outcomes were known, demonstrated this directly [9]. Whereas implantation and ongoing pregnancy/delivery rates per single euploid blastocyst transfers were more than 80% and 64.7%, respectively, only 40% of aneuploid blastocysts were implanted and there were no ongoing pregnancies.

Clinical outcomes following numerous RCTs have been mixed. Five early RCTs demonstrated improved clinical outcomes using different technologies and in various groups of patients with PGT-A [15–19]. Yang et al. examined the clinical pregnancy

rates after the transfer of a single blastocyst selected by PGT-A via aCGH plus morphology versus morphology alone in women under the age of 35 years with a tubal factor or male factor infertility undergoing their first cycle of IVF [15]. Among 103 women undergoing frozen embryo transfer (FET), the clinical pregnancy rate (70.9% vs. 45.8%, $p = 0.017$) and ongoing pregnancy rate (OPR) after 20 weeks gestation (69.1% vs. 41.7%, $p = 0.009$) were significantly higher in the PGT-A group.

Scott et al. evaluated the superiority of PGT-A with real-time quantitative polymerase chain reaction (qPCR) for embryo selection vs. traditional morphology [18]. Women in the biopsy group underwent PGT-A followed by fresh transfer of up to two euploid blastocysts vs. transfer of two untested blastocysts. Per embryo transferred, the IR (79.8% vs. 63.2%, $p = 0.002$) and sustained IR (66.4% vs. 47.9%, $p = 0.001$), or those embryos that went on to delivery, were higher in the PGT-A group. Per transfer, the clinical pregnancy (93.1% vs. 80.7%) and LBRs (84.7% vs. 67.5%) were similarly improved in the PGT-A group.

The BEST trial (Blastocyst Euploid Selective Transfer) was designed to compare single euploid transfer with the transfer of two untested embryos. This study enrolled women ≤42 years with at least two good-quality blastocysts [16]. In the PGT-A group, all available blastocysts underwent biopsy with qPCR analysis, and the single best-quality euploid blastocyst was transferred. Patients in the control group underwent transfer of the two best-untested blastocysts based on morphology alone. The OPR, defined as viable pregnancy ≥24 weeks gestation, was similar between groups (60.7% vs. 65.1%), whereas the rate of multiple gestations was significantly higher in the double embryo transfer group (53.4% vs. 0%). Women who underwent single euploid transfer were nearly twice as likely as those undergoing transfer of two untested embryos to have an ongoing singleton pregnancy (60.7% vs. 33.7%). A follow-up analysis of delivery outcomes demonstrated lower rates of preterm delivery (13% vs. 29%), low birth weight (11% vs. 33%), and neonatal intensive care unit stay (11% vs. 26%) in the single euploid transfer group [17]. Since the publication of the BEST trial, transfer of a single euploid embryo has become standard practice and is strongly recommended by the American Society for Reproductive Medicine (ASRM) due to equivalent OPRs with a stark decrease in the risk of multiple gestations and the associated obstetric risks [20].

A meta-analysis published in 2015 combined the results from these original RCTs, confirming increased clinical IRs (1.29, 95% Confidence Interval (CI) 1.15, 1.45) and sustained IRs (1.39, 95% CI 1.21, 1.60) in cycles utilizing PGT-A for identification of euploid embryos for transfer [21]. This same meta-analysis combined the results from eight observational studies, also finding increased clinical IRs (1.78, 95% CI 1.60–1.99) and sustained IRs (1.75, 95% CI 1.48–2.07) in the PGT-A groups. These early RCTs received much criticism for the fact that they included predominantly good prognosis patients with normal ovarian reserve who produced multiple high-quality blastocysts [22]. Consequently,

TABLE 25.1 Methods for Pre-implantation Genetic Testing for Aneuploidy (PGT-A) with Their Limitations and Additional Capabilities

Method ^a	Chromosome	Coverage	Resolution	Whole Chromosome Aneuploidy		Segmental Chromosome Aneuploidy		Abnormal Fertilization	Contamination	Parental Origin	Cost
				Full	Intermediate	Full	Intermediate				
Multicolour fluorescence in situ hybridization (mFISH)	Typically, 13, 16, 18, 21, 22, X, and Y	Probes mostly centromeric		✓		✗					Medium
Array comparative genomic hybridization (aCGH)	All 24 chromosomes	Approximately 3000 probes genome-wide		✓	✗	✓	✗				Medium
Array-based SNP genotyping and karyomapping with parental genotyping	Chromosomes 1–22 and X	1–5 Mb, depending on SNP coverage		✓	✗	✓	✗	✓	✓	✓	High
NGS-based copy number analysis	All 24 chromosomes	>10 Mb		✓	✓	✓	✓				Medium
Targeted NGS-based copy number and SNP analysis	All 24 chromosomes	Approximately 2500 SNP loci genome-wide		✓	✓	✓	✓	✓	✓	✗	Medium
NGS-based copy number and SNP analysis with parental genotyping	All 24 chromosomes	>10 Mb		✓	✓	✓	✓	✓	✓	✓	High

Note:

^a General guide only to the methodologies and not intended to be an accurate representation of specific commercially available tests.

patients who did not produce blastocysts were not randomized. Additionally, the aCGH and qPCR methods did not report on putative embryo mosaicism.

Rubio et al. sought to evaluate the effect of PGT-A in an older population (38–41 years old) with higher baseline rates of aneuploidy [19]. Women in the PGT-A group underwent cleavage-stage blastomere biopsy with aCGH analysis and subsequent fresh transfer of euploid blastocyst(s). The delivery rate per transfer was increased in the PGT-A group (52.9% vs. 24.2%, $p = 0.0002$) with a shorter time to pregnancy (7.7 vs. 14.9 weeks) and number of necessary transfers (1.8 vs. 3.7). Although this trial included cleavage-stage transfer, after the publication of a trial by Scott et al. that demonstrated a potential detrimental effect of blastomere biopsy, the field of PGT-A has shifted to almost exclusive use of trophectoderm biopsy at the expanded blastocyst stage [23].

In contrast to the Rubio trial, the STAR trial (Single-Embryo Transfer of Euploid Embryo), designed to compare outcomes when selecting embryos for transfer using PGT-A via NGS, found no significant improvement in clinical pregnancy or miscarriage rates [24]. This large RCT included women 25–40 years old, recruited from 34 clinics in the United States, the United Kingdom, and Australia, all with at least two blastocysts available for biopsy or transfer. The overall OPR per transfer (at ≥ 20 weeks' gestation) was similar between groups with no improvement in the PGT-A group. However, the OPR in the older subset of the cohort (35–40 years old) was significantly higher in the PGT-A group (50.8% vs. 37.2%, $p = 0.035$). Embryos with an intermediate copy number call, also called “mosaic” embryos, were deemed aneuploid and not eligible for transfer. Subsequent retrospective and non-selection studies have demonstrated that embryos with a mosaic report can perform similar to euploid, non-mosaic embryos and result in healthy live births [25, 26]. The inability to transfer mosaic embryos may have lowered the OPR in the group randomized to PGT-A.

To date, the largest RCT evaluating the impact of PGT-A via NGS technology on ART outcomes was published in 2021 [27]. This ambitious multicentre RCT randomized 1212 women aged 20 to 37 years who were undergoing their first IVF cycle and had three or more high-quality blastocysts. Women in the PGT-A group had their top three blastocysts biopsied. The primary outcome was cumulative LBR following sequential FETs until either live birth or all euploid (PGT-A group) or three best-untested embryos (control group) had been transferred. The cumulative LBR following a maximum of three transfers was similar between groups (PGT-A: 77.2% vs. control: 81.8%). However, more women in the control group required a second (192 vs. 119) or third (49 vs. 5) transfer. This study design likely underestimates the true cycle potential, as less than half of the available blastocysts were biopsied.

Given these mixed results, the use of PGT-A, therefore, remains controversial [28, 29]. One reason is that with highly effective vitrification protocols becoming increasingly routine, the emphasis on measuring IVF success rates has shifted to cumulative pregnancy and LBRs per cycle started or intention to treat. Clearly, any form of embryo selection in this context, including PGT-A, cannot change cumulative outcomes and embryo biopsy could potentially reduce them.

Also, the possibility that viable embryos could be discarded because of false positive results is a concern, particularly in poor prognosis patients [30]. To this end, non-selection studies, in which PGT-A tested embryos are transferred without prior

knowledge of the genetic test results, allow us to retrospectively analyse the outcomes of aneuploid transfers. The first such study was performed in 2012, examining both cleavage-stage and blastocyst biopsies analysed using SNP array technology [31]. A total of 255 morphologically selected embryos were chosen and biopsied shortly before transfer. Of 232 embryos with interpretable results, 99 were deemed aneuploid with four of these leading to live births, for a negative predictive value of 96%.

Going forward, a similar non-selection trial was performed to validate NGS technology [9]. The authors evaluated the transfer outcomes of 484 blastocysts in women aged 18–44 years. Biopsy results were analysed using NGS after knowledge of the clinical outcome was available, and were reported as euploid, whole chromosome aneuploid, whole chromosome mosaic, and segmental. Aneuploidy was detected in 102 embryos, leading to 41 (40.2%) positive pregnancy tests, 24 (23.5%) clinical pregnancies, and no sustained implantations beyond 13 weeks' gestation, for a 100% negative predictive value of aneuploid embryos.

An additional non-selection study from China divided PGT-A results first into euploid vs. aneuploid, and then into subcategories (euploid, euploid mosaic, euploid segmental, aneuploid, and aneuploid segmental) [32]. The LBR was 49.6% (67/135) in the euploid group vs. 7.5% (4/53) in the aneuploid group. Of the four live births in the aneuploid group, two were from embryos deemed to be uniformly aneuploid and two were thought to be segmental aneuploid. There were no differences in the LBRs between the uniformly euploid embryos vs. any other group except the uniformly aneuploid ($p < 0.0001$), although the segmental aneuploid group did trend towards worse outcomes.

Chromosome mosaicism

Unlike earlier methods used for PGT-A, including, for example, aCGH, low read depth, NGS-based copy number profiling has a linear relationship with chromosome copy number in the DNA amplified from the sample and increased resolution [33]. With multiple trophectoderm cell samples (typically 3–10 cells) biopsied at the blastocyst stage, this has enabled the identification of both whole and segmental chromosome aneuploidies with copy numbers ranging from those expected for trisomies or monosomies (full changes) to low or intermediate copy number changes (Figure 25.1). Intermediate copy number changes are generally interpreted as resulting from chromosome mosaicism between the cells of the biopsied trophectoderm cells, which can arise through non-disjunction and other mechanisms, including spindle abnormalities [34]. Whereas low copy number changes may be technical artefacts related to the amplification and NGS protocols used.

The clinical significance of intermediate or mosaic, whole or segmental copy number changes is not fully understood, since the PGT-A results are based on only a small sample of cells from the embryo and may not be fully representative, particularly of the inner cell mass lineage from which the fetus is formed. Indeed, transfer of mosaic embryos, following appropriate genetic counselling, has resulted in healthy live births [35–38]. Analysis of clinical outcomes following the transfer of more than 1000 mosaic blastocysts has confirmed that high-level mosaics (between 50% and 80%) have significantly lower ongoing pregnancy and LBRs compared with uniformly euploid blastocysts, whereas the outcomes with only low-level mosaicism (20%–50%) are similar [26]. Also, the number and type of aneuploidies present also affected the outcomes.

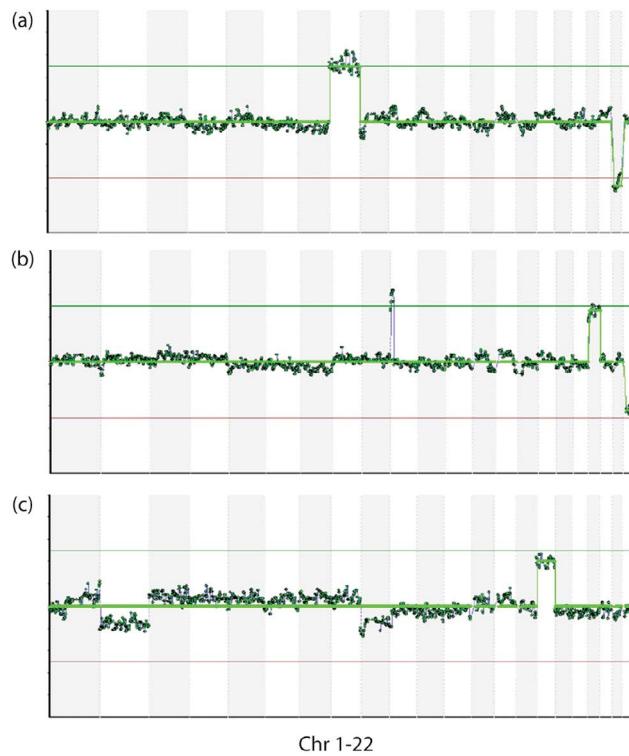


FIGURE 25.1 NGS-based chromosome copy number profiles for three trophectoderm biopsies following whole genome amplification, in a cycle from a patient aged 43 years, demonstrating the range of copy number changes which can be identified: (a) trisomy 8 and monosomy 22, (b) duplication of the terminal region of the short arm of chromosome 10 (p10dup), (c) mosaic loss of chromosomes 2 and 9 and trisomy 16. The dots represent the normalized distribution of fragments mapped to successive bins across each chromosome, and the bright green line indicates the best fit for two copies. The upper and lower green and red lines represent the theoretical displacement for three copies (trisomy) and one copy (monosomy), respectively.

Furthermore, a prospective non-selection trial confirmed that blastocysts with only low or moderate levels of mosaicism in the trophectoderm have equivalent developmental potential as those which are fully euploid [25]. Embryos with 20%–30% and 30%–50% aneuploid cells were labelled “low grade” and “medium grade” mosaic, respectively. A total of 897 embryos were available for transfer: 484 uniformly euploid, 282 low mosaic, and 131 moderate mosaic. The pregnancy, live birth, and miscarriage rates were similar across groups, demonstrating equivalent reproductive potential between euploid and low- to moderate-level mosaic embryos. The authors further performed karyotype analysis on 27 neonates born after mosaic embryo transfer and found that they all had normal karyotypes without persistence of the original mosaic findings.

The upper and lower thresholds for defining intermediate, mosaic changes from full aneuploidies will depend on the protocol used. By identifying meiotic errors in polar bodies and trophectoderm biopsies using SNP genotyping and karyomapping [39] in parallel with NGS-based PGT-A, it has recently been demonstrated that, with one exception, all female meiotic aneuploidies resulted

in copy number changes exceeding 70% of full changes in the corresponding trophectoderm biopsies or whole arrested embryos [40]. In contrast, most non-meiotic (presumed mitotic origin) aneuploidies had copy number changes ranging from 30% to 70%, although a minority exceeded the 70% threshold and may have resulted from chromosome mal-segregation in the first mitotic cleavage divisions. Furthermore, some samples originally reported as having mosaic, whole, or segmental chromosome copy number changes only were identified as having meiotic aneuploidies or, conversely, were euploid when reanalysed by karyomapping. Thus, one explanation for the differences in clinical outcomes between high- and low-level mosaic embryos may be the incidence of meiotic versus localized mitotic aneuploidies affecting the whole or only part of the embryo, respectively. Only a minority of otherwise euploid blastocysts have mosaic copy number changes, but the uncertain clinical significance of these abnormalities makes it good practice to require that patients have genetic counselling before considering these embryos for transfer [41].

Non-invasive PGT-A

The need to biopsy embryos for PGT-A increases the resources required and the cost for patients. Delaying biopsy to the blastocyst stage on days 5 to 7 post insemination, and limiting biopsy to good quality, clinical grade blastocysts, which can be considered for transfer, minimizes this. Biopsy could also potentially damage the embryos, though recent evidence demonstrates that limiting the number of trophectoderm cells removed (typically 3–10 cells) does not reduce either implantation or OPRs following transfer of euploid blastocysts [9]. Nevertheless, non-invasive PGT-A (niPGT-A) would eliminate the possibility of damage, broaden access, and lower costs. Reports that cell-free DNA is present in blastocoel fluid and spent culture medium at the blastocyst stage [42, 43] therefore have led to numerous reports of niPGT-A protocols which aim to amplify and test this cell-free DNA [44–46].

Ideally, niPGT-A should be minimally invasive, accurate, and efficient [47]. Blastocentesis, in which blastocoel fluid is aspirated by penetrating the trophectoderm layer with a sharp pipette to sample cell-free DNA, presumably from dead cells in the blastocoel cavity, is clearly less invasive than biopsy of multiple trophectoderm cells, and is already used in some laboratories to collapse the blastocoel cavity to improve survival after vitrification [42, 48]. In contrast, sampling of spent culture medium is only “invasive” to the extent that embryos need to be cultured individually and, to avoid maternal contamination, carefully denuded of cumulus cells, washed extensively, the media changed before sampling, and culture extended to at least day 6 post insemination [45].

For niPGT-A, various protocols have been used to optimize whole genome amplification from fragmented cell-free DNA, and use the amplified DNA for NGS-based copy number analysis. However, copy number analysis alone does not allow the origin of the cell-free DNA to be tested, and a recent study, using whole genome methylation sequencing, demonstrated that differentially methylated regions characteristic of blastocyst, cumulus cell, and oocyte/polar body DNA could be detected [49]. This confirms a previous report that DNA from the second polar body can persist to the blastocyst stage and could potentially give false results at a chromosomal and single gene level [50]. Protocols that include analysis of genome-wide SNP markers, for example, may therefore be necessary to avoid false negative results caused by maternal contamination [9].

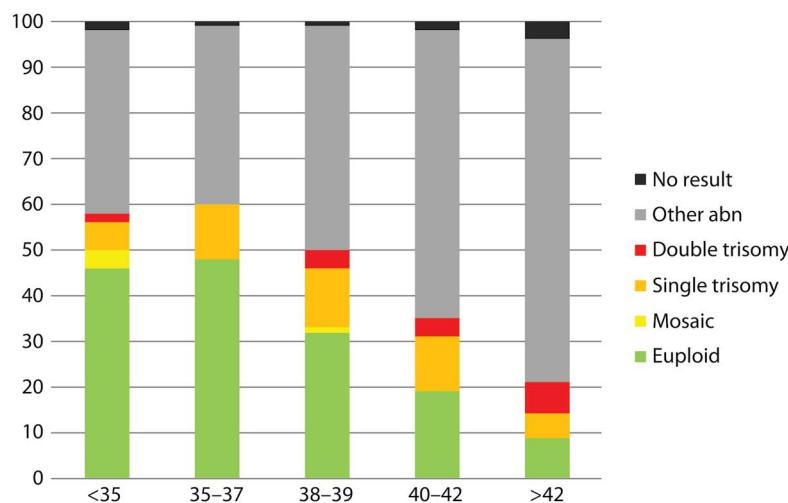


FIGURE 25.2 Distribution of euploid, full copy single trisomies, double trisomies, mosaic, and other complex aneuploidies based on the results of single trophectoderm biopsy of all biopsied blastocysts [14]. The groupings are based on the chromosome complement observed in an analysis of retained products of conception following single blastocyst transfer [53].

The accuracy of niPGT-A tested in concordance with corresponding trophectoderm biopsies, whole blastocysts and, in some cases, the isolated inner cell mass has varied widely [51]. However, a recent multicentre prospective study of concordance between cell-free DNA and trophectoderm biopsies from 1301 human blastocysts in which the medium was changed on day 4 and harvested ≥ 40 hours later demonstrated a concordance of 78% with a 12% and 8% false positive and false negative rate, respectively, 12.5% maternal contamination and failed in about 12% of samples [45]. Thus, for clinics willing to change their embryology protocols, niPGT-A may be useful for prioritizing blastocysts for embryo transfer.

Prospects for integration into routine clinical practice

The methods used for PGT-A continue to evolve, and several now include SNP markers to improve accuracy and extend diagnostic capabilities, allowing the detection of, for example, abnormally fertilized embryos and contamination. Alternatively, niPGT-A offers the possibility of avoiding embryo biopsy and can be used for prioritizing embryos for transfer but with a lower accuracy. PGT-A remains challenging, with uncertainties around intermediate chromosome copy number changes and the requirement for genetic counselling [52], and costs are still high. Nevertheless, PGT-A is now in widespread use and has a range of clinical and laboratory benefits (Table 25.2). Most importantly, these include optimizing pregnancy rates following single embryo transfer to avoid the complications of multiple pregnancies, reducing the risk of miscarriage and abnormal pregnancy, and improved clinical and laboratory management. The use of PGT-A has confirmed the high incidence not only of single aneuploidies but also multiple aneuploidies and other abnormalities known to result in pregnancy loss and which increase exponentially with maternal age [14, 53] (Figure 25.2). Accurate and cost-effective methods of PGT-A are therefore essential to improve clinical outcomes and increase our understanding of the causes of infertility and IVF failure beyond chromosome aneuploidy.

TABLE 25.2 Clinical and Laboratory Benefits of Combined Time-Lapse Analysis, Vitrification, and PGT-A at the Blastocyst Stage

- High implantation and clinical pregnancy rates and low miscarriage rates with single euploid, vitrified, warmed blastocyst transfers
- Cryo-storage of euploid blastocysts only
- Reduces the risk of chromosomally abnormal pregnancy or live birth
- Improves clinical management and embryology standards
- Enables patients to make informed decisions

References

1. McKinlay Gardner R, Amor D (eds.). Gardner and Sutherland's Chromosome Abnormalities and Genetic Counseling, 5th ed. Oxford University Press (OUP), 2018.
2. Moorthie S, Blencowe H, Darlison MW, Gibbons S, Lawn JE, Mastroiacovo P, et al. Chromosomal disorders: Estimating baseline birth prevalence and pregnancy outcomes worldwide. *J Community Genet.* 2018 Oct 26;9(4):377–86.
3. Nagaoka SI, Hassold TJ, Hunt PA. Human aneuploidy: Mechanisms and new insights into an age-old problem. *Nat Rev Genet.* 2012 Jun 18;13(7):493–504.
4. Ishihara O, Jwa SC, Kuwahara A, Katagiri Y, Kuwabara Y, Hamatani T, et al. Assisted reproductive technology in Japan: A summary report for 2017 by the Ethics Committee of the Japan Society of Obstetrics and Gynecology. *Reprod Med Biol.* 2020 Jan 1;19(1):3–12.
5. Verlinsky Y, Cieslak J, Ivakhnenko V, Lifchez A, Strom C, Kuliev A. Birth of healthy children after preimplantation diagnosis of common aneuploidies by polar body fluorescent in situ hybridization analysis. *Preimplantation Genetics Group. Fertil Steril.* 1996 Jul;66(1):126–9.
6. Munné S, Alikani M, Tomkin G, Grifo J, Cohen J. Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertil Steril.* 1995 Aug;64(2):382–91.
7. Mastenbroek S, Twisk M, van Echten-Arends J, Sikkema-Raddatz B, Korevaar JC, Verhoeve HR, et al. In vitro fertilization with preimplantation genetic screening. *N Engl J Med.* 2007 Jul 5;357(1):9–17.

8. Handyside AH. 24-chromosome copy number analysis: A comparison of available technologies. *Fertil Steril.* 2013 Sep;100(3):595–602.
9. Tiegs AW, Tao X, Zhan Y, Whitehead C, Kim J, Hanson B, et al. A multicenter, prospective, blinded, nonselection study evaluating the predictive value of an aneuploid diagnosis using a targeted next-generation sequencing-based preimplantation genetic testing for aneuploidy assay and impact of biopsy. *Fertil Steril.* 2021 Mar 1;115(3):627–37.
10. Rienzi L, Gracia C, Maggiulli R, Labarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: Systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update.* 2017 Mar 1;23(2):139–55.
11. Glujsovsky D, Quintero Retamar AM, Alvarez Sedo CR, Ciapponi A, Cornelisse S, Blake D. Cleavage-stage versus blastocyst-stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev.* 2022 May 19;2022(5).
12. Whitney JB, Schiwe MC, Anderson RE. Single center validation of routine blastocyst biopsy implementation. *J Assist Reprod Genet.* 2016 Nov 20;33(11):1507–13.
13. Barash OO, Ivani KA, Willman SP, Rosenbluth EM, Wachs DS, Hinckley MD, et al. Association between growth dynamics, morphological parameters, the chromosomal status of the blastocysts, and clinical outcomes in IVF PGS cycles with single embryo transfer. *J Assist Reprod Genet.* 2017 Aug 30;34(8):1007–16.
14. Gorodeckaja J, Neumann S, McCollin A, Ottolini CS, Wang J, Ahuja K, et al. High implantation and clinical pregnancy rates with single vitrified-warmed blastocyst transfer and optional aneuploidy testing for all patients. *Hum Fertil (Camb).* 2020 Oct 1;23(4):256–67.
15. Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, et al. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: Results from a randomized pilot study. *Mol Cytogenet.* 2012 May 2;5(1):24.
16. Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B, et al. In vitro fertilization with single euploid blastocyst transfer: A randomized controlled trial. *Fertil Steril.* 2013 Jul;100(1):100–7.e1.
17. Forman EJ, Hong KH, Franasiak JM, Scott RT. Obstetrical and neonatal outcomes from the BEST Trial: Single embryo transfer with aneuploidy screening improves outcomes after in vitro fertilization without compromising delivery rates. *Am J Obstet Gynecol.* 2014 Feb;210(2):157.e1–157.e6.
18. Scott RT, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, et al. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: A randomized controlled trial. *Fertil Steril.* 2013 Sep;100(3):697–703.
19. Rubio C, Bellver J, Rodrigo L, Castillón G, Guillén A, Vidal C, et al. In vitro fertilization with preimplantation genetic diagnosis for aneuploidies in advanced maternal age: A randomized, controlled study. *Fertil Steril.* 2017 May;107(5):1122–9.
20. Practice Committee of the American Society for Reproductive Medicine and the Practice Committee for the Society for Assisted Reproductive Technologies. Guidance on the limits to the number of embryos to transfer: A committee opinion. *Fertil Steril.* 2021 Sep 1;116(3):651–4.
21. Dahdouh EM, Balayla J, García-Velasco JA. Comprehensive chromosome screening improves embryo selection: A meta-analysis. *Fertil Steril.* 2015;104(6):1503–12.
22. Orvieta R, Gleicher N. Should preimplantation genetic screening (PGS) be implemented to routine IVF practice? *J Assist Reprod Genet.* 2016 Nov;33(11):1445–8.
23. Scott RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: A randomized and paired clinical trial. *Fertil Steril.* 2013 Sep;100(3):624–30.
24. Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, et al. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: A multicenter randomized clinical trial. *Fertil Steril.* 2019;112(6).
25. Capalbo A, Poli M, Rienzi L, Girardi L, Patassini C, Fabiani M, et al. Mosaic human preimplantation embryos and their developmental potential in a prospective, non-selection clinical trial. *Am J Hum Genet.* 2021 Dec 2;108(12):2238–47.
26. Viotti M, Victor AR, Barnes FL, Zouves CG, Besser AG, Grifo JA, et al. Using outcome data from one thousand mosaic embryo transfers to formulate an embryo ranking system for clinical use. *Fertil Steril.* 2021 May 1;115(5):1212–24.
27. Yan J, Qin Y, Zhao H, Sun Y, Gong F, Li R, et al. Live birth with or without preimplantation genetic testing for aneuploidy. *N Engl J Med.* 2021 Nov 25;385(22):2047–58.
28. Rosenwaks Z, Handyside AH. Is preimplantation genetic testing for aneuploidy an essential tool for embryo selection or a costly “add-on” of no clinical benefit? *Fertil Steril.* 2018 Aug;110(3):351–2.
29. Rosenwaks Z, Handyside AH, Fiorentino F, Gleicher N, Paulson RJ, Schattman GL, et al. The pros and cons of preimplantation genetic testing for aneuploidy: Clinical and laboratory perspectives. *Fertil Steril.* 2018 Aug;110(3):353–61.
30. Paulson RJ. Hidden in plain sight: The overstated benefits and underestimated losses of potential implantations associated with advertised PGT-A success rates. *Hum Reprod.* 2020 Mar 27;35(3):490–3.
31. Scott RT, Ferry K, Su J, Tao X, Scott K, Treff NR. Comprehensive chromosome screening is highly predictive of the reproductive potential of human embryos: A prospective, blinded, nonselection study. *Fertil Steril.* 2012 Apr;97(4):870–5.
32. Wang L, Wang X, Liu Y, Ou X, Li M, Chen L, et al. IVF embryo choices and pregnancy outcomes. *Prenat Diagn.* 2021 Dec 1;41(13):1709–17.
33. Fiorentino F, Biricik A, Bono S, Spizzichino L, Cotroneo E, Cottone G, et al. Development and validation of a next-generation sequencing-based protocol for 24-chromosome aneuploidy screening of embryos. *Fertil Steril.* 2014 May;101(5):1375–82.
34. Chatzimeletiou K, Morrison EE, Prapas N, Prapas Y, Handyside AH. Spindle abnormalities in normally developing and arrested human preimplantation embryos in vitro identified by confocal laser scanning microscopy. *Hum Reprod.* 2005 Mar;20(3):672–82.
35. Greco E, Minasi MG, Fiorentino F. Healthy babies after intrauterine transfer of mosaic aneuploid blastocysts. *N Engl J Med.* 2015 Nov 19;373(21):2089–90.
36. Fragouli E, Alfarawati S, Spath K, Babariya D, Tarozzi N, Borini A, et al. Analysis of implantation and ongoing pregnancy rates following the transfer of mosaic diploid-aneuploid blastocysts. *Hum Genet.* 2017 Jul 9;136(7):805–19.
37. Spinella F, Fiorentino F, Biricik A, Bono S, Ruberti A, Cotroneo E, et al. Extent of chromosomal mosaicism influences the clinical outcome of in vitro fertilization treatments. *Fertil Steril.* 2018 Jan;109(1):77–83.
38. Victor AR, Tyndall JC, Brake AJ, Lepkowsky LT, Murphy AE, Griffin DK, et al. One hundred mosaic embryos transferred prospectively in a single clinic: Exploring when and why they result in healthy pregnancies. *Fertil Steril.* 2019 Feb 1;111(2):280–93.
39. Handyside AH, Harton GL, Mariani B, Thornhill AR, Affara N, Shaw M-A, et al. Karyomapping: A universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. *J Med Genet.* 2010 Oct 1;47(10):651–8.
40. Handyside AH, McCollin A, Summers MC, Ottolini CS. Copy number analysis of meiotic and postzygotic mitotic aneuploidies in trophectoderm cells biopsied at the blastocyst stage and arrested embryos. *Prenat Diagn.* 2021 Apr 1;41(5):525–35.

41. Leigh D, Cram DS, Rechitsky S, Handyside A, Wells D, Munne S, et al. PGDIS position statement on the transfer of mosaic embryos 2021. *Reprod Biomed Online*. 2022 Jul 1;45(1):19–25.
42. Gianaroli L, Magli MC, Pomante A, Crivello AM, Cafueri G, Valerio M, et al. Blastocentesis: A source of DNA for preimplantation genetic testing. Results from a pilot study. *Fertil Steril*. 2014 Dec;102(6):1692–9.e6.
43. Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: Preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. *Fertil Steril*. 2016 Nov;106(6):1312–8.
44. Xu J, Fang R, Chen L, Chen D, Xiao J-P, Yang W, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci U S A*. 2016;113(42):11907–12.
45. Rubio C, Navarro-Sánchez L, García-Pascual CM, Ocalí O, Cimadomo D, Venier W, et al. Multicenter prospective study of concordance between embryonic cell-free DNA and trophectoderm biopsies from 1301 human blastocysts. *Am J Obstet Gynecol*. 2020 Nov 1;223(5):751.e1–751.e13.
46. Chen L, Sun Q, Xu J, Fu H, Liu Y, Yao Y, et al. A non-invasive chromosome screening strategy for prioritizing in vitro fertilization embryos for implantation. *Front Cell Dev Biol*. 2021 Aug 9; 9:708322.
47. Handyside AH. Noninvasive preimplantation genetic testing: Dream or reality? *Fertil Steril*. 2016 Nov;106(6):1324–5.
48. Magli MC, Albanese C, Crippa A, Tabanelli C, Ferraretti AP, Gianaroli L. Deoxyribonucleic acid detection in blastocoelic fluid: A new predictor of embryo ploidy and viable pregnancy. *Fertil Steril*. 2019 Jan;111(1):77–85.
49. Chen Y, Gao Y, Jia J, Chang L, Liu P, Qiao J, et al. DNA methylome reveals cellular origin of cell-free DNA in spent medium of human preimplantation embryos. *J Clin Invest*. 2021 Jun 1;131(12):e146051.
50. Ottolini CS, Rogers S, Sage K, Summers MC, Capalbo A, Griffin DK, et al. Karyomapping identifies second polar body DNA persisting to the blastocyst stage: Implications for embryo biopsy. *Reprod Biomed Online*. 2015 Dec;31(6):776–82.
51. Li J, Liu Y, Qian Y, Zhang D. Noninvasive preimplantation genetic testing in assisted reproductive technology: Current state and future perspectives. *J Genet Genomics*. 2020 Dec 20;47(12):723–6.
52. Capalbo A, Poli M, Jalas C, Forman EJ, Treff NR. On the reproductive capabilities of aneuploid human preimplantation embryos. *Am J Hum Genet*. 2022 Sep 1;109(9):1572–81.
53. Segawa T, Kuroda T, Kato K, Kuroda M, Omi K, Miyauchi O, et al. Cytogenetic analysis of the retained products of conception after missed abortion following blastocyst transfer: A retrospective, large-scale, single-centre study. *Reprod Biomed Online*. 2017 Feb;34(2):203–10.

26

DIAGNOSIS OF ENDOMETRIAL RECEPTIVITY, EMBRYO-ENDOMETRIAL DIALOGUE, AND ENDOMETRIAL MICROBIOME

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Introduction

Implantation is the first major physical embryo–maternal interaction. Successful implantation requires synchronized and bidirectional communication followed by embryo adhesion and invasion of the decidualized endometrium [1]. The human endometrium is a complex tissue composed of predominantly epithelial and stromal cells, which are cyclically regulated by steroid hormones (oestrogens and progesterone [P]). Endometrial receptivity is a self-limited period when the endometrium is transiently receptive to implantation of a blastocyst, a process that is mediated by immune cells, cytokines, growth factors, chemokines, adhesion molecules, and several other molecules through different pathways (for review, see [2–4]). A receptive endometrium, a viable euploid blastocyst, and an exquisitely coordinated cross-communication between them are required for successful implantation and invasion of the underlying maternal endometrium.

In this chapter, we discuss the molecular characterization of endometrial receptivity through transcriptomics with special attention to its clinical translation. We also examine embryo–endometrial dialogue that plays an important role in transmitting information from mother to embryo during peri-implantation. We close by discussing the endometrial microbiota and its impact on reproduction. Together, we provide a complete view of the diverse events that take place in the pre-conceptional space and how they may impact embryo implantation.

Molecular diagnosis of endometrial receptivity

Advances in endometrial biology fall closely on the heels of the genomics revolution. Early endometrial transcriptomic profiling studies identified the window of implantation (WOI) during natural cycles and yielded four simultaneous reports on the human secretory endometrium transcriptome (for review, see [5]). Later efforts characterized transcriptomic profiles across the menstrual cycle, under controlled ovarian stimulation (COS), and in patients with refractory cycles (for review, see [5]). Studies on the endometrial transcriptome have since proliferated (for review, see [6]), and additional work has identified transcriptomic differences relating to endometrial pathologies and factors that may affect fertility [7–13]. These findings catalysed the acceptance of the importance of molecular endometrial factors in fertility and health in addition to anatomical factors.

Our group first defined human endometrial receptivity in terms of its molecular (transcriptomic) signature. The signature comprised 238 differentially expressed genes, which, when

combined with a computational algorithm, provided a predictor to classify endometrial samples into proliferative (PRO), pre-receptive (PRE), receptive (R), or post-receptive (POST) phases [14]. This work prompted a new approach to assessing endometrial receptivity beyond classical histological dating. Further, our 2020 study confirmed the transcriptomic signature across endometrial phases at a single-cell resolution [15]. By analysing more than 70,000 individual cells falling into six distinct endometrial cell types, we identified that transcriptomic activation in the epithelial cells opens the WOI [15].

This molecular evidence base provided the impetus for applying endometrial receptivity analysis (ERA) to diagnose and treat recurrent implantation failure (RIF) of endometrial origin [16]. The goal of this approach is to time embryo transfer while the endometrium is receptive (i.e. personalized embryo transfer [pET]). ERA revealed critical information about the WOI. The WOI lasts only 30–36 hours, and, depending on the patient, it can occur at different moments during the cycle [usually between five and ten days after luteinizing hormone (LH) surge in natural cycles (LH+6 to LH+9) or between three and eight days of P exposure in hormonal replacement therapy cycles (P+4 to P+7)] [17]. Because the WOI is not the same for all women, results of the ERA after five full days of P exposure (P+5; the standard WOI) reveal how the embryo transfer should be personalized for each case (Figure 26.1).

We tested whether ERA could produce accurate and reproducible results across the menstrual cycle by using the histological gold standard. To achieve this, data for 49 endometrial biopsies were assessed using the quadratic weighted Kappa index [18]. We also determined whether ERA results were reproducible across cycles in the same individual by analysing biopsy pairs collected 29–40 months apart under the same conditions. No inter-cycle variation was detected [18]; however, specific variables could affect the endometrium, increasing the probability of a displaced WOI. One factor is body mass index (BMI). In a prospective cohort study stratifying patients by BMI, WOI displacement was more common among those with a BMI of >30 kg/m² than in those with a BMI of <30 kg/m² [19].

The final proof of concept to demonstrate the utility of the endometrial receptivity testing is how personalizing the embryo transfer impacts in the clinical outcome (for review, see [20]). To date, 27 publications describe clinical outcomes for ERA-guided pET; these studies occurred at different fertility centres, encompass specific clinical indications, and report different outcomes (Table 26.1). These studies show how synchronizing the transfer of a viable blastocyst with a receptive endometrium, can increase the success rate in patients with previous implantation failures.

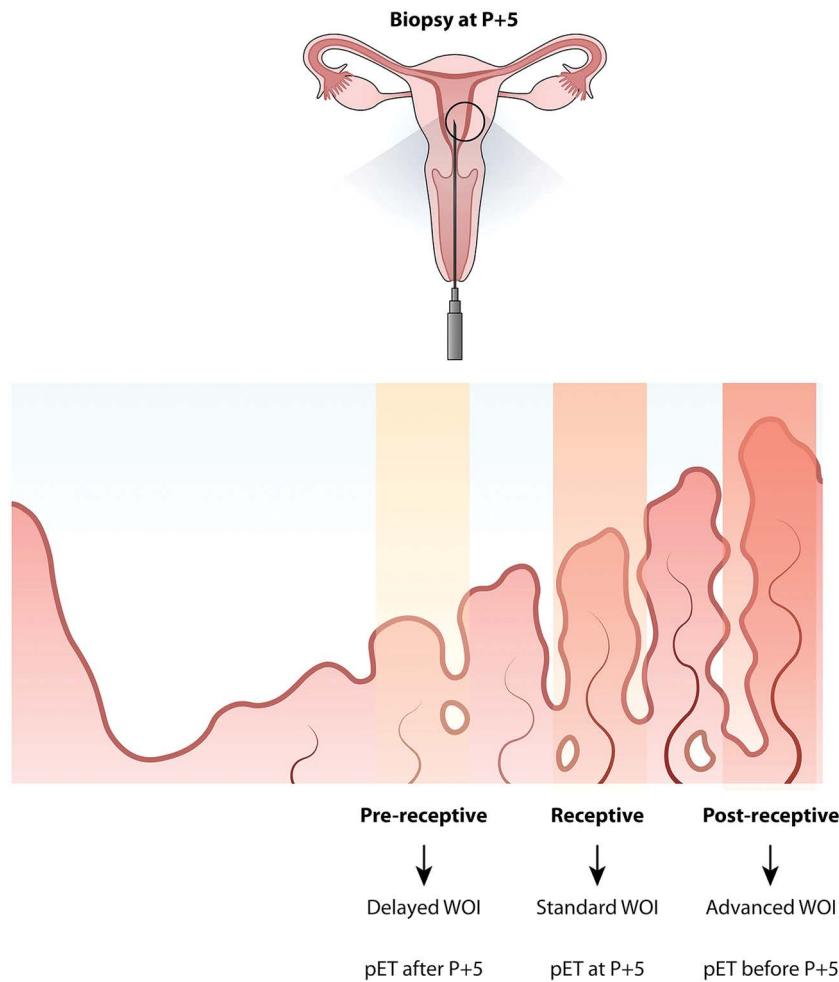


FIGURE 26.1 Representation of different displacements of the WOI. ERA identifies endometrial status from an endometrial biopsy collected during the standard WOI (at P+5). If there is no displacement, the ERA result will be receptive. If a pre-receptive result is obtained, it means that the WOI is delayed, so pET should be performed after P+5. However, if a post-receptive result is obtained, the WOI has already passed, and pET should be performed before P+5.

Embryo–endometrial dialogue

Intercellular communication is established during cell-to-cell contact that permits the release and uptake of different chemicals, hormones, or growth factors [21]. This process is essential for multicellular organisms and for relationships between unicellular organisms and the environment or their hosts [22]. Signalling during implantation represents the first major physical embryo–maternal interaction and requires synchronized, bidirectional communication [1, 23–25]. Human pre-implantation embryos produce soluble ligands and receive signals through their receptors in an autocrine/paracrine fashion, including soluble factors of maternal origin [26]. Numerous ligands (mainly cytokines and growth factors) are produced by the human endometrium during the receptive phase [27]. An embryo–maternal “cross-talk” or molecular dialogue is therefore postulated to exist during the peri-implantation period. These signalling pathways are highly complex and are often described as regulatory “circuits” [28]. Recently, evidence has emerged to support the existence of cross-talk between the mother and the embryo.

Mother-to-embryo communication

Extracellular vesicles (EVs) are critical factors in intercellular communication [29, 30]. EVs function by direct interaction with cell surface receptors or by transmission of their contents via endocytosis, phagocytosis, or fusion with target cell membranes. Recipient cell specificity seems to be driven by receptors present in cells and EVs [30, 31]. Communication between the mother and embryo appears to be mediated through EVs that are present in the endometrial fluid [32–35] and released by endometrial epithelial cells [32, 33, 36, 37].

Our laboratory demonstrated effective communication between the mother and the pre-implantation embryo through EVs. EVs are present in human endometrial fluid [33], and exosomes containing microRNA (miRNA) has-miR-30d are actively transferred from endometrial epithelial cells to embryo trophoblast cells, where the miRNA is internalized. Murine embryos treated with a synthetic analogue of human miR-30d show altered gene expression, with higher expression of genes encoding molecules involved in embryo adhesion, such as *Itgb3*, *Itga7*,

TABLE 26.1 ERA Clinical Publications. Scientific Publications Showing the Clinical Outcome Obtained after Performing pET Guided by ERA

Study type	Year	Title	Authors	Journal	Sample size	Main findings
RCT	2020	A five-year multicentre randomized controlled trial comparing personalized, frozen and fresh blastocyst transfer in IVF	Simon C. et al.	Reproductive BioMedicine Online, 2020; 41(3):402–415	458 patients with blastocyst transfer at first appointment were randomized to pET guided by ERA, FET, or fresh ET	Per ITT: No differences except cumulative PR that was significantly higher in the pET (93.6%) vs. FET (79.7%) ($p = 0.0005$) and fresh ET (80.7%) ($p = 0.0013$). Per protocol: LBR at first embryo transfer were 56.2% in PET versus 42.4% in FET ($P = 0.09$), and 45.7% in fresh embryo transfer groups ($P = 0.17$). Cumulative LBR were 71.2% in PET versus 55.4% in FET ($P = 0.04$), and 48.9% in fresh ET ($P = 0.003$). PR in PET, FET and fresh embryo transfer arms were 72.5% versus 54.3% ($P = 0.01$) and 58.5% ($P = 0.05$), respectively. IR were 57.3% versus 43.2% ($P = 0.03$), and 38.6% ($P = 0.004$), respectively. Obstetrical outcomes, type of delivery, and neonatal outcomes were similar in all groups.
Prospective	2013	The endometrial receptivity array for diagnosis and personalized embryo transfer (pET) as a treatment for patients with repeated implantation failure	Ruiz-Alonso M. et al.	Fertil Steril. 2013; 100(3):818–24	RIF group: n = 85 Control group: n = 25	WOI was displaced in 25.9% of patients in the RIF group vs. 12% in the control group. RIF patients after pET reached PR 51.7% and IR 38.5% that is similar to good prognosis patients
	2021	Role of endometrial receptivity array for implantation failure in in-vitro fertilization & intracytoplasmic sperm injection	Nafees et al.	Biomedica. 2021; 37(4):220–226.	Patients with ≥1 IF (n = 16)	Displaced WOI in 25% of patients. PR of 75%.
	2021	Routine endometrial receptivity array in first embryo transfer cycles does not improve live birth rate	Riestenberg C. et al.	Fertil Steril. 2021; 115(4):1001–1006.	ERA group n = 147 Standard ET group n = 81	Displaced WOI in 59.2% of ERA group patients. LBR was not different between pET and standard ET group (56.6% vs. 56.5%).
Retrospective	2014	What a difference two days make: “personalized” embryo transfer (pET) paradigm: a case report and pilot study	Ruiz-Alonso M. et al.	Hum Reprod. 2014; 29(6):1244–7.	Case report and Series with 1–6 failed transfers (n = 17)	Case report: clinical case of successful pET after seven previous failed IVF attempts. Case series: After pET these patients reached 60% PR vs 19% PR after ET in a non-receptive endometrium diagnosed by ERA.
	2015	Endometrial receptivity array: Clinical application	Mahajan N.	J Hum Reprod Sci. 2015; 8(3):121–9.	RIF group (n = 80) Control group (n = 93)	In RIF Indian population, WOI displacement was 27.5% vs. 15% in control non-RIF group ($P = 0.04$). Both groups have pET guided by ERA, reaching similar outcomes: RIF: OPR 42.4% and IR 33% vs. Non-RIF: OPR 56% and IR 39% ($p > 0.1$).
	2017	Efficacy of the endometrial receptivity array for repeated implantation failure in Japan: A retrospective, two-centers study	Hashimoto T. et al.	Reprod Med Biol. 2017; 16(3):290–296.	RIF group (n = 50)	In RIF Japanese population, WOI displacement was found in 24% of patients. RIF patients with displaced or non-displaced WOI reached similar outcomes after pET: PR 35.3% in receptive (R) patients vs. 50% in non-receptive (NR) patients ($p = 0.9$).

(Continued)

TABLE 26.1 ERA Clinical Publications. Scientific Publications Showing the Clinical Outcome Obtained after Performing pET Guided by ERA (Continued)

Study type	Year	Title	Authors	Journal	Sample size	Main findings
Retrospective	2017	Window of implantation (WOI) transcriptomic stratification reveals different endometrial subsignatures associated with live birth and biochemical pregnancy	Diaz-Gimeno P. et al.	Fertil Steril. 2017; 108(4):703–710.e3.	Fertile donors (n = 79) ERA patients (n = 771)	Ongoing PR ranged from 76.9% and 80% in the late pre-receptive and receptive (R), respectively, versus 33.3% when ET is performed in late-receptive. The biochemical pregnancy was 7.7% and 6.6% in late pre-receptive and R, respectively, but 50% when ET was performed in late-receptive.
	2018	The role of the endometrial receptivity array (ERA) in patients who have failed euploid embryo transfers	Tan J. et al.	J Assist Reprod Genet. 2018; 35(4): 683–92.	ERA group with ≥1 previously failed euploid transfer (n = 88)	WOI displacement in 22.5% of patients. RIF patients with displaced or non-displaced WOI reached similar outcomes after pET. In the group of RIF patients with euploid transfer, IR and OPR were apparently higher after correcting the WOI in NR vs. R patients (76.5 vs. 53.8% and 64.7 vs. 42.3%, respectively) although not statistically significant.
	2018	Does the endometrial receptivity array really provide pET?	Bassil R. et al.	J Assist Reprod Genet. 2018; 35(7):1301–1305.	ERA group with 0–2 failed transfer (n = 53) Standard ET group with 0–2 failed transfer (n = 503)	WOI displacement in 64.15% of ERA groups patients. No statistically significant differences in PR between pET and ET group (39% vs. 35.2%)
	2018	WOI is significantly displaced in patients with adenomyosis with previous implantation failure as determined by endometrial receptivity assay	Mahajan N. et al.	Journal of human reproductive sciences. 2018; 11(4):353.	Adenomyosis group (n=36) Control group (n = 338)	In adenomyosis, WOI was significantly displaced (47.2%) vs. controls (21.6%) ($p < 0.001$) The incidence of RIF in adenomyosis was 66.6% versus 34.9% in controls ($P < 0.001$). PR after pET in adenomyosis group was 62.5%.
	2019	pET Helps in Improving IVF/ICSI Outcomes in Patients with Recurrent Implantation Failure (RIF)	Patel JA. et al.	J Hum Reprod Sci. 2019; 12(1):59–66.	RIF group (n = 248)	WOI displacement in 17.7% of RIF patients. RIF patients with displaced or non-displaced WOI reached similar outcomes after pET Ongoing PR 41.7% vs. 42.9% ($p = 0.93$).
	2019	Endometrial Receptivity Analysis – a tool to increase an implantation rate in assisted reproduction.	Hromadova L. et al.	Ceska Gynekol. 2019; 84(3):177–183.	ERA group (n = 85)	WOI displacement in 36.5% of patients. PR after pET in NR patients was 69.2%.
	2019	What is the clinical impact of the endometrial receptivity array in PGT-A and oocyte donation cycles?	Neves AR. et al.	J Assist Reprod Genet. 2019; 36:1901	pET group with ≥1 previously failed euploid transfer (n = 24) or with ≥2 previously failed donor transfer (n = 32) Standard ET group with ≥1 previously failed euploid transfer (n = 119) or ≥2 previously failed donor transfer (n = 158)	After euploid embryo transfer no differences in pET vs ET groups were found (IR 55.6% vs. 65% and PR 58.3% vs. 70.6%). Significant lower PR (34.4% vs. 65.2%; $p = 0.001$) in donor pET group vs. donor standard ET group. RIF patients with displaced or non-displaced WOI reached similar outcomes after pET in both euploid and donor arms.

(Continued)

TABLE 26.1 ERA Clinical Publications. Scientific Publications Showing the Clinical Outcome Obtained after Performing pET Guided by ERA (Continued)

Study type	Year	Title	Authors	Journal	Sample size	Main findings
Retrospective	2020	Evaluation of the endometrial receptivity assay and the pre-implantation genetic test for aneuploidy in overcoming RIF	Cozzolino M. et al.	J Assist Reprod Genet. 2020; 37(12):2989–2997.	Moderate RIF group: (n = 2110) Severe RIF group (n = 488)	Patients with euploid embryo transferred in the moderate RIF group, had higher IR and ongoing PR than those without PGT-A. The use of the ERA test did not appear to significantly improve clinical outcomes in either group.
	2020	Comparing endometrial receptivity array to histologic dating of the endometrium in women with a history of implantation failure	Cohen AM. et al.	Syst Biol Reprod Med. 2020; 66(6):347–354.	RIF group (n = 97)	WOI was displaced in 47.4% of patients. The concordance between ERA and histological dating was 40.0%.
	2020	Does pET based on ERA improve the outcomes in patients with thin endometrium and RIF in self versus donor programme?	Selvaraj P. et al	Gynecological Research and Obstetrics 6.3 (2020): 076–080.	RIF self oocyte ERA (n = 179) RIF self non-ERA (n = 180) RIF donation ERA (n = 181) RIF donation non-ERA (n = 182)	RIF patients with displaced (22.5%) or non-displaced WOI reached similar clinical PR after pET (26.7% vs. 22.5%) ($p = 0.66$). Displaced WOI in 35–39% of patients. Clinical outcome not statistically different between patients with self-oocytes with and without ERA (due to embryo factor) but significant higher in ovum donation patients with ERA (CPR 59.4%) than without ERA (CPR 43.4%)
	2021	Clinical utility of the endometrial receptivity analysis in women with prior failed transfers	Eisman LE. et al.	J Assist Reprod Genet. 2021; 38(3):645–650.	ERA group with ≥ 1 previously failed transfer (n = 131) Control group (n=91)	WOI was displaced in 45% of patients with ≥ 1 failed transfer, 40% of patients with ≥ 3 previously failed transfer and 52% of control patients. The pregnancy outcomes did not differ between women with ≥ 1 prior failed ET and controls. In women with ≥ 3 prior failed ETs, there was a lower ongoing pregnancy/LBR (28% vs. 54%, $p = 0.046$).
	2021	Evaluation of Pregnancy Outcomes of Vitrified-Warmed Blastocyst Transfer before and after Endometrial Receptivity Analysis in Identical Patients with RIF	Kasahara Y. et al.	Fertility & Reproduction. 2020; 3(2):35–41	RIF group (n = 95)	In RIF patients, comparison of previous ET and pET demonstrate a significant increase in PR for pET per patient and cycle (5.3% vs. 62.8%, 4.4% vs. 47.9%, respectively). PR, IR at the first pET were significantly higher in patients with displaced WOI vs. non-displaced.
	2021	The use of propensity score matching to assess the benefits of the endometrial receptivity analysis in frozen embryo transfers	Bergin K. et al.	Fertil Steril. 2021; 116(2):396–403.	ERA group (n = 133) Non-ERA group (n = 353)	No statistically significant differences were found between ERA and non-ERA group (LBR 49.62% vs. 54.96%)
	2021	Do clinical outcomes differ for day-5 versus day-6 single embryo transfers controlled for endometrial factor?	Stankewicz T. et al.	Reprod Biomed Online. 2021 Nov 18:S1472-6483(21)00581-2.	Day 5 blastoc: 183 Day 6 blastoc: 77	Clinical outcomes were similar when transferring day-5 blastocysts versus day-6 blastocysts: PR 75.4% and 70.1% ($P = 0.465$); IR 67.8% and 63.6% ($P = 0.476$); and OPR 57.9% and 58.4% ($P = 0.728$).

(Continued)

TABLE 26.1 ERA Clinical Publications. Scientific Publications Showing the Clinical Outcome Obtained after Performing pET Guided by ERA (Continued)

Study type	Year	Title	Authors	Journal	Sample size	Main findings
Retrospective	2022	Role of endometrial receptivity array in RIF	Samadhiya R. et al.	Fertility Science and Research 8.2 (2021): 180.	RIF patients (n = 34)	WOI displacement in 38.2% of patients. PR and IR after pET in the NR group, achieved 50% and 45.5%, similar to the 55.4% obtained in general patients (non RIF).
	2022	Comparison of the Effectiveness of Endometrial Receptivity Analysis (ERA) to Guide pET with Conventional Frozen Embryo Transfer in 281 Chinese Women with RIF	Jia Y. et al.	Medical Science Monitor: International Medical Journal of Experimental and Clinical Research 28 (2022): e935634–e935634.	RIF ERA group (n = 140) RIF Non-ERA group (n = 141)	The ERA test identified 35% of samples as R and 65% as NR in the ERA group. Higher Clinical PR and IR were found in the ERA group than in the non-ERA group (Clinical PR with ERA 50% vs 24.8% without ERA; P < 0.01), while no significant differences were detected between the two groups in terms of miscarriage rates (P > 0.05).
Case report	2014	Live birth after embryo transfer in an unresponsive thin endometrium	Cruz F. & Bellver J.	Gynecol Endocrinol. 2014; 30(7):481–4.	Case report	WOI found in an endometrium with 3.5mm with subsequent live birth achieved after pET.
	2018	Different Endometrial Receptivity in Each Hemiuterus of a Woman With Uterus Didelphys and Previous Failed Embryo Transfers	Carranza F. et al	J Hum Reprod Sci. 2018; 11(3):297–299.	Case report	ERA showed Receptivity in the right-sided hemiuterus while the left-sided hemiuterus was NR. Live birth achieved after pET in the right-sided hemiuterus.
	2019	Why results of endometrial receptivity assay testing should not be discounted in RIF?	Simrandeep K. et al.	The Onco Fertility Journal. 2019; 2(1): 46–49.	Cases report (n = 3)	Three severe cases of RIF patients; two of them had a previous ERA performed at a different centre, but pET not followed, resulting in failure. Once pET was implemented, successful clinical pregnancy was achieved in both patients.
	2019	The Reproductive Outcomes for the Infertile Patients with RIFs May Be Improved by Endometrial Receptivity Array Test	Ota T. et al.	Journal of Medical Cases. 2019; 10(5), 138–140.	Case report	Patient who achieved pregnancy with pET guided by ERA after 11 previous failed attempts.

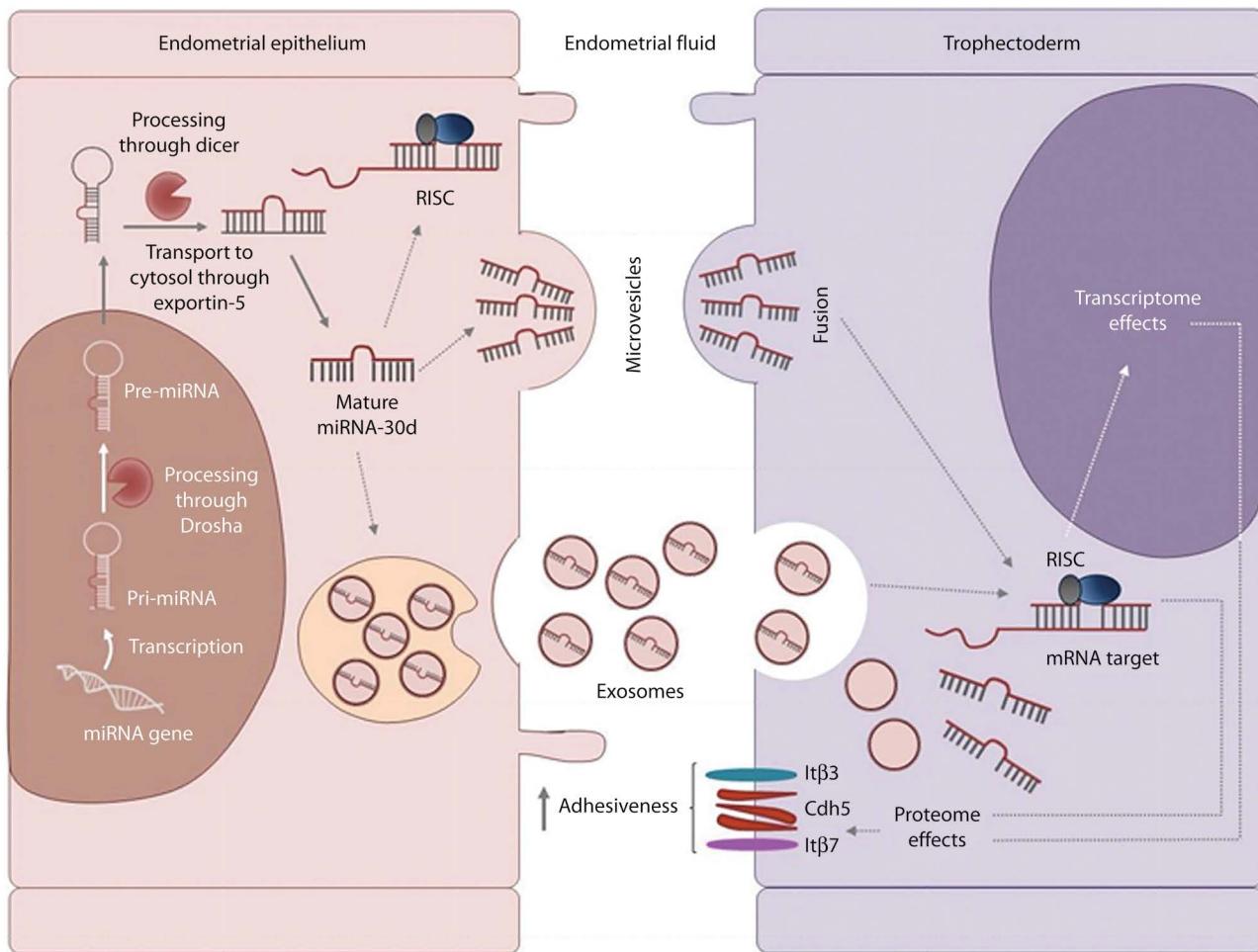


FIGURE 26.2 Schematic representation of cross-talk between the mother and embryo. Endometrial epithelial cells encapsulate miRNAs in EVs. Specifically, miRNA-30d secreted by epithelial cells are encapsulated in EVs and internalized in trophoblastic cells that produce effects to increase the adhesiveness of the embryo (from [32], reprinted with permission).

and *Cdh5*, highlighting the importance of miR-30d transference via exosomes [32] (Figure 26.2). EV proteomes are regulated by steroid hormones, and exosomes produced during the receptive phase may potentially impact embryo implantation. For example, exosomes are internalized by human trophoblastic cells, enhancing their adhesive capacity partially through the focal adhesion kinase cascade [37].

DNA transmission between cells may also occur via EVs. Single-stranded DNA and double-stranded DNA are present in different types of vesicles [38], and horizontal transfer of EV DNA is a possible new mechanism for the transfer of genetic material across species [39]. Exosomes and micro-vesicles are also transporters of mitochondrial DNA (mtDNA) and may therefore transmit altered mtDNA [40]. Vertical transmission of maternal mtDNA cargo to the embryo may be a mechanism to modulate embryo bioenergetics during the peri-conceptional period [41].

Embryo-to-mother communication

The pre-implantation embryo releases specific molecular effectors to the extracellular milieu, which influence endometrial cell gene expression (e.g. trypsin released by embryos before

implantation raises calcium signalling in endometrial epithelial cells) [42–44]. Decidualized stromal cells recognize incompetent embryos and act as biosensors of embryo quality by assessing these embryo-derived soluble factors that are involved in implantation. Decidualized stromal cells of patients with recurrent miscarriages cannot discriminate between low- and high-quality human embryos [42, 45]. Therefore, disruption of this biosensing process can result in recurrent miscarriage, and natural killer cells play an important role as modulators of these biosensors [46].

To analyse molecules released by embryos, laboratories can use culture medium from *in vitro* fertilization (IVF) cycles, allowing for the characterization of paracrine/autocrine processes. Secretion of miRNAs during early embryo developmental stages may mediate such dialogue between an embryo and the mother. miRNAs are detected at a much higher scale at the blastocyst stage, indicating that this type of signalling begins soon after blastulation in a period concomitant with endometrial invasion [47].

Embryos also produce EVs that participate in cross-talk with the endometrium [35, 48] and in autocrine regulation [49]. The presence of specific embryonic exosomes significantly increases

from the cleavage stage to the blastocyst stage [50]. Endometrial cells may take up different types of vesicles that contain miRNAs and other molecules [27, 32]. In *in vitro* models, EVs isolated from a trophectoderm cell line stimulate the proliferation of endothelial cells, showing that EVs can act as potential regulators of endometrial angiogenesis [36]. Additional investigations of this potential communication between embryos and implantation sites are needed to better understand the processes underlying implantation.

The endometrial microbiome

The existence of an endometrial microbiome was demonstrated recently through the use of culture-independent techniques (fingerprinting, targeted amplification, DNA microarrays, metagenomic sequencing, etc.) [51]. The most common method for bacterial profiling is 16S rRNA gene sequencing. This gene is conserved in every bacterium and contains numerous species-specific hypervariable regions that can be used like “fingerprints” to identify bacterial taxa based on reference sequences [52, 53]. This technique can detect minute amounts of microbial DNA in the environmental or in biological specimens and has helped reveal the indigenous microbiota of numerous body sites [54], including those classically considered sterile, such as the uterine cavity.

The female reproductive tract possesses a microbiota continuum that increases in diversity and decreases in abundance from the outer to the inner organs [55]. Thus, the endometrial microbiota, like others in the upper reproductive tract, has a low biomass, which is estimated to be 100–10,000 times lower than the bacterial load in the vagina [55]. The uterine cavity may be colonized via several mechanisms, including sexual activity, gynaecological procedures, hematogenous spread of oral or respiratory bacteria, and translocation of gut or urinary microbiota to the reproductive tract [56, 57]. The most plausible route of endometrial colonization is the ascension of bacteria from the vagina due to its close proximity and direct communication with the uterine cavity. This hypothesis is supported by the similarity between bacterial profiles found in the endometrium and vagina of women at different ages, the presence of polymicrobial biofilms adhered to the uterine lining of women with vaginal infections, the results of animal studies of bacterial translocation, and the increasing convergence of endometrial and vaginal microbiota across the lifespan [58–61].

Increasing evidence shows that *Lactobacillus* is the most common genus in the endometrium of reproductive-aged women. Consistent with the vagina, the dominance of *Lactobacillus* spp. is considered the reproductive tract physiological flora [58, 62–65] (Figure 26.3). Other bacterial genera, such as *Gardnerella*, *Prevotella*, *Streptococcus*, *Clostridium*, *Bacteroides*, *Atopobium*, etc., have also been detected in the endometrium [58, 61, 65–67]. However, despite global similarities between the bacterial composition of endometrial and vaginal microbiota, some studies analysing paired endometrial and vaginal samples collected from reproductive-aged women showed that the microbiota of these two body sites are not identical in every woman. This finding shows that potential pathogenic bacteria may reside in the endometrium and are absent in the vagina and vice versa [58, 62].

Deviations from a *Lactobacillus*-dominated endometrial microbiota are associated with gynaecological conditions, including endometrial polyps [68], endometrial cancer [69], endometriosis [55, 70], menorrhagia and dysmenorrhea [71], and infertility [64, 72].

Specifically, chronic endometritis (CE; persistent inflammation of the endometrium caused by a subclinical bacterial infection with common pathogens) is significantly associated with RIF and recurrent pregnancy loss (RPL) [73–75]. Comparative studies showed worse reproductive outcomes in RIF [implantation rate (IR): 15% CE versus 46% no CE] and RPL patients [live birth (LB) rate: 7% before treatment versus 56% after treatment] with concomitant CE than in patients without CE [76, 77]. Importantly, antibiotic treatment in RIF patients improves IR, clinical pregnancy rate (CPR), and LB rate after eliminating CE, and clinical results in patients with cured CE were comparable with those in patients without CE [78].

The reproductive tract microbiota may fluctuate in response to endogenous and exogenous factors, including hormones [57, 79]. A study conducted in 392 RIF patients revealed that 44.9% had a non-*Lactobacillus*-dominated endometrial microbiota with high abundance of *Gardnerella*, *Atopobium*, *Streptococcus*, and *Prevotella* [72]. The content of *Lactobacillus* in endometrial fluid increases with follicular development, starting with <50% of *Lactobacillus* after menstruation and gradually increasing to an average of >70% in the luteal phase [72]. In the context of IVF, the endometrial microbiota may change following COS cycles and P supplementation [67, 71], with decreased *Lactobacillus* content and increased abundance of reproductive tract pathogens, such as *Atopobium*, *Escherichia*, and *Prevotella* [67]. Because of this, when managing patients with infertility undergoing assisted reproductive technology (ART) treatment, the endometrial microbiota should be assessed during the WOI in a mock cycle before embryo transfer to accurately assess the microbial environment that the embryo may encounter during implantation. Failure to assess the microbiome under these conditions may result in misdiagnosis of the endometrial microbiota in these patients.

Several studies analysed the association between composition of the endometrial microbiota and clinical results in ART patients, with variable results [80]. Patients with positive culture for pathogens such as anaerobic bacteria, Enterobacteriaceae, *Enterococcus* spp., *Escherichia coli*, *Haemophilus* spp., *Klebsiella pneumoniae*, *Staphylococcus* spp., and *Streptococcus* spp. in the uterine cavity at the time of embryo transfer had lower IR and CPR and higher clinical miscarriage rates than patients with negative cultures [81–86]. Results from molecular studies using 16S rRNA sequencing that analysed the whole endometrial microbiota have led to consideration of the endometrial microbiota from an ecological point of view. The impact of the endometrial microbiota on IVF outcomes was first demonstrated in a cohort of 35 RIF patients in which a *Lactobacillus*-dominated microbiota (>90% *Lactobacillus*) was associated with increased LB rate, while the presence of pathogenic bacteria, to the detriment of *Lactobacillus*, was significantly associated with reproductive failure [58]. These results were corroborated in a recent international observational prospective study conducted in 342 patients that confirmed the significant association between composition of the endometrial microbiota and reproductive outcomes after receiving pET at the time of the maximum endometrial receptivity, as determined by ERA [65]. Women with a higher abundance of lactobacilli are more likely to achieve a live birth, while the pathogenic profile associated with reproductive failure (no pregnancy, biochemical pregnancy, or clinical miscarriage) consisted of *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus*, and *Streptococcus* [65] (Figure 26.3).

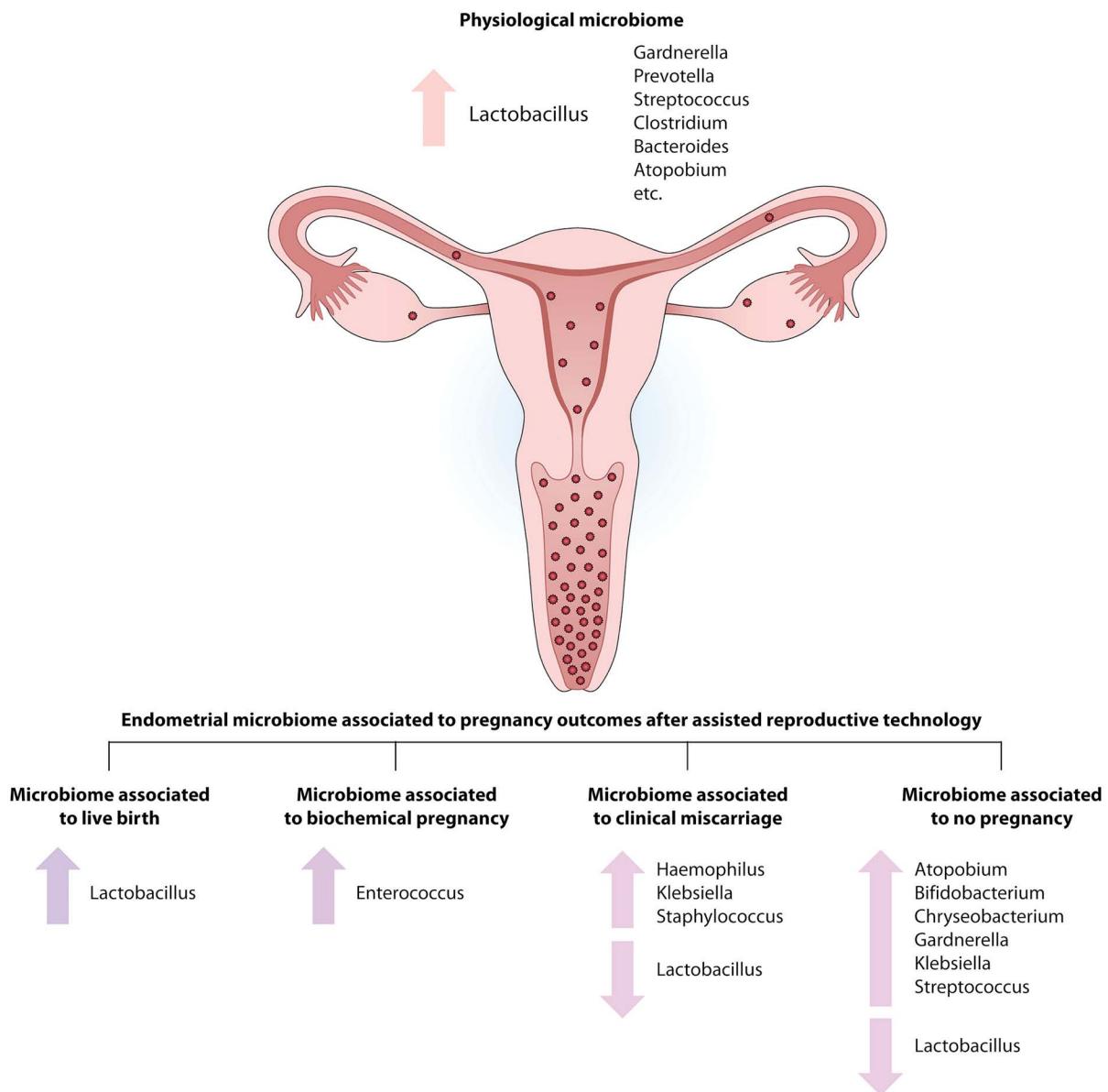


FIGURE 26.3 Endometrial microbiome composition in physiological and pathological conditions. The endometrial microbiota in reproductive-aged women is mainly composed of *Lactobacillus* species under physiological conditions, although other bacterial taxa are identified at lower abundances. Pathological conditions, such as infertility, are characterized by a shift in the endometrial microbiota towards increased abundance of bacterial pathogens. In the context of IVF, dominance of *Lactobacillus* is associated with successful ART, while implantation failure or pregnancy loss is associated with a concomitant decrease in *Lactobacillus* and increase in bacterial pathogens.

The mechanisms behind the detrimental effects of dysbiotic microbiota in embryo implantation are still unknown, but the secretion of bacterial metabolites may disrupt the endometrial epithelial barrier, causing exacerbated immune and inflammatory responses, deregulation of metalloproteinases and other structural proteins relevant for embryo implantation, and secretion of pathogenic molecules that may produce toxicity in the ready-to-implant uterus. However, while these hypotheses warrant further testing, endometrial microbiota composition can be considered as a complement to ERA in cases of infertility of endometrial origin.

Conclusion

New transcriptomics-based molecular methods to assess endometrial receptivity have been introduced into the clinic in recent years, providing us with molecular tools to time the WOI and improve IVF success. Several other “omics” approaches are now being applied to unravel the complex process of endometrial receptivity. Although some technical limitations still exist, we believe that integrative sciences are the future of diagnosing the correct timing for embryo implantation. Furthermore, the application of these new technologies should be used to improve

our knowledge of endometrial receptivity, providing additional knowledge on critical dialogue between the embryo and endometrium and the endometrial microbiota composition. This knowledge will enable the discovery of the main causes of implantation failure and open new avenues of investigation into interceptive molecules to aid in the diagnosis and treatment strategies to improve embryo implantation.

References

- Moreno I, Capalbo A, Mas A, Garrido-Gomez T, Roson B, Poli M, et al. The human periconceptional maternal-embryonic space in health and disease. *Physiol Rev.* 2023;103(3):1965–2038.
- Kämmerer U, von Wolff M, Markert UR. Immunology of human endometrium. *Inmunobiology.* 2004;209:569–74.
- Giudice LC. Implantation and endometrial function. In: Molecular Biology in Reproductive Medicine. Fauzer BCJM (ed.). London, UK: Parthenon Publishing Group, 1999.
- Dimitriadis E, White CA, Jones R, Salomonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update.* 2005;11:613–30.
- Ruiz-Alonso M, Blesa D, Simon C. The genomics of the human endometrium. *BBA Mol Basis Dis.* 2012;1822:1931–1942.
- Díaz-Gimeno P, Ruiz-Alonso M, Blesa D, Simon C. Transcriptomics of the human endometrium. *Int J Dev Biol.* 2014;58(2–4):127–137.
- Tapia A, Gangi LM, Zegers-Hochschild F, Balmaceda J, Pommer R, Trejo L, et al. Differences in the endometrial transcript profile during the receptive period between women who were refractory to implantation and those who achieved pregnancy. *Hum Reprod.* 2008;23:340–51.
- Koler M, Achache H, Tsafrir A, Smith Y, Revel A, Reich R. Disrupted gene pattern in patients with repeated in vitro fertilization (IVF) failure. *Hum Reprod.* 2009;24:2541–48.
- Altmac S, Martínez-Conejero JA, Salumets A, Simon C, Horcajadas JA, Stavreus-Evers A. Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. *Mol Hum Reprod.* 2010;16:178–87.
- Matsuzaki S. DNA microarray analysis in endometriosis for development of more effective targeted therapies. *Front Biosci (Elite Ed).* 2011;3:1139–53.
- Habermann JK, Bundgen NK, Gemoll T, Hautaniemi S, Lundgren C, Wangsa D, et al. Genomic instability influences the transcriptome and proteome in endometrial cancer subtypes. *Mol Cancer.* 2011;10:132.
- Garcia-Velasco JA, Fassbender A, Ruiz-Alonso M, Blesa D, Thomas DH, Simon C. Is endometrial receptivity transcriptomics affected in women with endometriosis? A pilot study. *Reprod Biomed Online.* 2015;31(5):647–654.
- Comstock IA, Diaz-Gimeno P, Cabanillas S, Bellver J, Sebastian Leon P, Shah M, et al. Does an increased body mass index affect endometrial gene expression patterns in infertile patients? A functional genomics analysis. *Fertil Steril.* 2017;107(3):740–748.e2.
- Díaz-Gimeno P, Horcajadas J, Martínez-Conejero J, Esteban F, Alama P, Pellicer A, et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril.* 2011;95:50–60.
- Wang W, Vilella F, Alama P, Moreno I, Mignardi M, Isakova A, et al. Single-cell transcriptomic atlas of the human endometrium during the menstrual cycle. *Nat Med.* 2020;26:1644–53.
- Ruiz-Alonso M, Blesa D, Díaz-Gimeno P, Gómez E, Fernández-Sánchez M, Carranza F, et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. *Fertil Steril.* 2013 Sep;100(3):818–24.
- Rincon A, Clemente-Ciscar M, Gomez E, Marin C, Valbuena D, Simon C. That is the real length of the window of implantation (WOI) in humans? In: European Society of Human Reproduction and Embryology; July 1–July 4, 2018; Barcelona, Spain. Abstract P-477.
- Díaz-Gimeno P, Ruiz-Alonso M, Blesa D, Bosch N, Martínez-Conejero JA, Alamá P, et al. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. *Fertil Steril.* 2013;99(2):508–517.
- Bellver J, Marín C, Lathi RB, Murugappan G, Labarta E, Vidal C, et al. Obesity affects endometrial receptivity by displacing the window of implantation. *Reprod Sci.* 2021;28(11):3171–3180.
- Ruiz-Alonso M, Valbuena D, Gomez C, Cuzzi J, Simon C. Endometrial receptivity analysis (ERA): Data versus opinions. *Hum Reprod Open.* 2021;2021(2):hoab011.
- Raposo G, Stoorvogel W. Extracellular vesicles: Exosomes, microvesicles, and friends. *J Cell Biology.* 2013;200(4):373–83.
- Yáñez-Mó M, Siljander PRM, Andreu Z, Zavec AB, Borrás FE, Buzás EI, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles.* 2015 May 14;4(0):27066.
- Hardy K, Spanos S. Growth factor expression and function in the human and mouse preimplantation embryo. *J Endocrinol.* 2002;172(2):221–36.
- Richter KS. The importance of growth factors for preimplantation embryo development and in-vitro culture. *Curr Opin Obstetrics Gynecol.* 2008;20(3):292–304.
- Martín JC, Jasper MJ, Valbuena D, Meseguer M, Remoh J, Pellicer A, et al. Increased adhesiveness in cultured endometrial-derived cells is related to the absence of moesin expression. *Biol Reprod.* 2000 Nov;63(5):1370–6.
- Thouas GA, Dominguez F, Green MP, Vilella F, Simon C, Gardner DK. Soluble ligands and their receptors in human embryo development and implantation. *Endocr Rev.* 1996;17(1):92–130.
- Simon C, Greening DW, Bolumar D, Balaguer N, Salamonsen LA, Vilella F. Extracellular vesicles in human reproduction in health and disease. *Endocr Rev.* 2018;39(3):292–332.
- Kaye P. Preimplantation growth factor physiology. *Rev Reprod.* 1997;2(2):121–7.
- Yuana Y, Oosterkamp TH, Bahatyrova S, Ashcroft B, Rodriguez PG, Bertina RM, et al. Atomic force microscopy: A novel approach to the detection of nanosized blood microparticles. *J Thromb Haemost.* 2010;8(2):315–23.
- Andaloussi SE, Mäger I, Breakefield XO, Wood MJA. Extracellular vesicles: Biology and emerging therapeutic opportunities. *Nat Rev Drug Discov.* 2013;12(5):347–57.
- Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol.* 2014 Oct 11;30(1):255–89.
- Vilella F, Moreno-Moya JM, Balaguer N, Grasso A, Herrero M, Martínez S, et al. Hsa-miR-30d, secreted by the human endometrium, is taken up by the pre-implantation embryo and might modify its transcriptome. *Development.* 2015;142(18):3210–21.
- Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL, et al. Endometrial exosomes/microvesicles in the uterine microenvironment: A new paradigm for embryo-endometrial cross talk at implantation. *PLoS One.* 2013;8(3):e58502.
- Burns G, Brooks K, Wildung M, Navakanitworakul R, Christenson LK, Spencer TE. Extracellular vesicles in luminal fluid of the ovine uterus. *PLoS One.* 2014;9(3):e90913.
- Burns GW, Brooks KE, Spencer TE. Extracellular vesicles originate from the conceptus and uterus during early pregnancy in sheep. *Biol Reprod.* 2016;94(3):56.
- Bidarimath M, Khalaj K, Kridli RT, Kan FWK, Koti M, Tayade C. Extracellular vesicle mediated intercellular communication at the porcine maternal-fetal interface: A new paradigm for conceptus-endometrial cross-talk. *Sci Rep.* 2017 Jan 12;7:40476.

37. Greening DW, Nguyen HPT, Elgass K, Simpson RJ, Salamonsen LA. Human endometrial exosomes contain hormone-specific cargo modulating trophoblast adhesive capacity: Insights into endometrial-embryo interactions. *Biol Reprod.* 2016;94(2):Article 38, 1–15.
38. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: A novel biomarker in cancer detection. *Cell Res.* 2014 Apr 8;24(6):766–9.
39. Kawamura Y, Yamamoto Y, Sato T, Ochiya T. Extracellular vesicles as trans-genomic agents: Emerging roles in disease and evolution. *Cancer Sci.* 2017;108(5):824–30.
40. Guescini M, Guidolin D, Vallorani L, Casadei L, Gioacchini AM, Tibollo P, et al. C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction. *Exp Cell Res.* 2010;316(12):1977–84.
41. Bolumar D, Amadoz A, Moreno I, Marín C, Juan AD, Simon C, et al. Endometrial mitochondrial DNA secreted in extracellular vesicles: A novel maternal mechanism modulating embryo bioenergetics. *Fertil Steril.* 2020;114(3):e34.
42. Weimar CHE, Kavelaars A, Brosens JJ, Gellersen B, Vreeden-Elbertse JMT, de Heijnen CJ, et al. Endometrial stromal cells of women with recurrent miscarriage fail to discriminate between high- and low-quality human embryos. *PLoS One.* 2012;7(7):e41424.
43. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo Quality. *Biol Reprod.* 2014;91(4):Article 98, 1–8.
44. Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, et al. Uterine selection of human embryos at implantation. *Sci Rep.* 2014;4(1):3894.
45. Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, et al. Natural selection of human embryos: Decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. *PLoS One.* 2010;5(4):e10258.
46. Orlando J, Coulam C. Is superfertility associated with recurrent pregnancy loss? *Am J Reprod Immunol.* 2014;72(6):549–54.
47. Cimadomo D, Rienzi L, Giancani A, Alviggi E, Dusi L, Canipari R, et al. Definition and validation of a custom protocol to detect miRNAs in the spent media after blastocyst culture: Searching for biomarkers of implantation. *Hum Reprod.* 2019;34(9):1746–61.
48. Simon B, Bolumar D, Amadoz A, Jimenez-Almazán J, Valbuena D, Vilella F, et al. Identification and characterization of extracellular vesicles and its DNA cargo secreted during murine embryo development. *Genes (Basel).* 2020;11(2):203.
49. Desrochers LM, Bordeleau F, Reinhart-King CA, Antonyak MA, Cerione RA. Microvesicles provide a mechanism for intercellular communication by embryonic stem cells during embryo implantation. *Nat Commun.* 2016;7(1):11.
50. Giacomini E, Vago R, Sanchez AM, Podini P, Zarovni N, Murdica V, et al. Secretome of in vitro cultured human embryos contains extracellular vesicles that are taken up by the maternal side. *Sci Rep.* 2017;7(1):5210.
51. Mor A, Driggers PH, Segars JH. Molecular characterization of the human microbiome from a reproductive perspective. *Fertil Steril.* 2015;104(6):1344–50.
52. Patel JB. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Mol Diagn.* 2001;6(4):313–21. doi: [10.1054/modi.2001.29158](https://doi.org/10.1054/modi.2001.29158).
53. Hamady M, Knight R. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. *Genome Res.* 2009;19(7):1141–52. doi: [10.1101/gr.085464.108](https://doi.org/10.1101/gr.085464.108).
54. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature.* 2012;486(7402):207–14.
55. Chen C, Song X, Wei W, Zhong H, Dai J, Lan Z, et al. The Microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat Commun.* 2017;8:875.
56. Baker JM, Chase DM, Herbst-Kralovetz MM. Uterine Microbiota: Residents, tourists, or invaders?. *Front Immunol.* 2018;9:208.
57. Laniewski P, Ilhan ZE, Herbst-Kralovetz MM. The microbiome and gynaecological cancer development, prevention and therapy. *Nat Rev Urol.* 2020;17(4):232–50.
58. Moreno I, Codoñer FM, Vilella F, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol.* 2016;215(6), 684–703.
59. Swidsinski A, Verstraeten H, Loening-Baucke V, Swidsinski S, Mendling W, Halwani Z. Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. *PLoS One.* 2013;8(1):e53997.
60. Racicot K, Cardenas I, Wünsche V, et al. Viral infection of the pregnant cervix predisposes to ascending bacterial infection. *J Immunol.* 2013;191(2):934–41.
61. Wang J, Li Z, Ma X, et al. Translocation of vaginal microbiota is involved in impairment and protection of uterine health. *Nat Commun.* 2021;12:4191.
62. Mitchell CM, Haick A, Nkwopara E, Garcia R, Rendi M, Agnew K, et al. Colonization of the upper genital tract by vaginal bacterial species in nonpregnant women. *Am J Obstet Gynecol.* 2015;212(5):611.e1–19.
63. Tao X, Franasiak JM, Zhan Y, Scott RT, Rajchel J, Bedard J, et al. Characterizing the endometrial microbiome by analyzing the ultra-low bacteria from embryo transfer catheter tips in IVF cycles: Next generation sequencing (NGS) analysis of the 16S ribosomal Gene. *Hum Microbiome J.* 2017;3:15–21.
64. Kyono K, Hashimoto T, Nagai Y, Sakuraba Y. Analysis of endometrial Microbiota by 16S ribosomal RNA Gene sequencing among infertile patients: A single-center pilot study. *Reprod Med Biol.* 2018;17:297–306.
65. Moreno I, Garcia-Grau I, Perez-Villaroya D, et al. Endometrial microbiota composition is associated with reproductive outcome in infertile patients. *Microbiome.* 2022;10(1):1.
66. Verstraeten H, Vilchez-Vargas R, Desimpel F, et al. Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. *PeerJ.* 2016;4:e1602.
67. Carosso A, Revelli A, Gennarelli G, Canosa S, Cosma S, Borella F, et al. Controlled ovarian stimulation and progesterone supplementation affect vaginal and endometrial Microbiota in IVF cycles: A pilot study. *J Assist Reprod Genet.* 2020;37:2315–26.
68. Fang RL, Chen LX, Shu WS, Yao SZ, Wang SW, Chen YQ. Barcoded sequencing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. *Am J Transl Res.* 2016;8(3):1581–92.
69. Walther-António MR, Chen J, Multinu F, Hokenstad A, Distad TJ, Cheek EH, et al. Potential contribution of the uterine microbiome in the development of endometrial cancer. *Genome Med.* 2016;8(1):122.
70. Khan KN, Fujishita A, Masumoto H, Muto H, Kitajima M, Masuzaki H, et al. Molecular detection of intrauterine microbial colonization in women with endometriosis. *Eur J Obstet Gynecol Reprod Biol.* 2016;199:69–75.
71. Pelzer ES, Willner D, Buttini M, Huygens F. A role for the endometrial microbiome in dysfunctional menstrual bleeding. *Antonie Van Leeuwenhoek.* 2018;111(6):933–43.
72. Kadogami D, Nakaoaka Y, Morimoto Y. Use of a vaginal probiotic suppository and antibiotics to influence the composition of the endometrial microbiota. *Reprod Biol.* 2020;20(3):307–14.
73. Kitaya K, Takeuchi T, Mizuta S, Matsubayashi H, Ishikawa T. Endometritis: New time, new concepts. *Fertil Steril.* 2018;110(3):344–50.
74. Cicinelli E, Matteo M, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, et al. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. *Reprod Sci.* 2014;21(5):640–47.

75. Cincinelli E, Matteo M, Tinelli R, Lepera A, Alfonso R, Indraccolo U, et al. Prevalence of chronic endometritis in repeated unexplained implantation failure and the IVF success rate after antibiotic therapy. *Hum Reprod.* 2015;30(2):323–30.
76. Johnston-MacAnally EB, Hartnett J, Engmann LL, Nulsen JC, Sanders MM, Benadiva CA. Chronic endometritis is a frequent finding in women with recurrent implantation failure after in vitro fertilization. *Fertil Steril.* 2010;93(2):437–41.
77. McQueen DB, Bernardi LA, Stephenson MD. Chronic endometritis in women with recurrent early pregnancy loss and/or fetal demise. *Fertil Steril.* 2014;101(4):1026–30.
78. Vitagliano A, Saccardi C, Noventa M, Di Spiezio Sardo A, Saccone G, Cincinelli E, et al. Effects of chronic endometritis therapy on in vitro fertilization outcome in women with repeated implantation failure: A systematic review and meta-analysis. *Fertil Steril.* 2018;110(1):103–112.e101.
79. Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UM, Zhong X, et al. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med.* 2012;4(132):132ra152.
80. Toson B, Simon C, Moreno I. The endometrial microbiome and its impact on human conception. *Int J Mol Sci.* 2022;23(1):485.
81. Egbase PE, al-Sharhan M, al-Othman S, al-Mutawa M, Udo EE, Grudzinskas JG. Incidence of microbial growth from the tip of the embryo transfer catheter after embryo transfer in relation to clinical pregnancy rate following in-vitro fertilization and embryo transfer. *Hum Reprod.* 1996;11(8):1687–89.
82. Egbase PE, Udo EE, Al-Sharhan M, Grudzinskas JG. Prophylactic antibiotics and endocervical microbial inoculation of the endometrium at embryo transfer. *Lancet.* 1999;354(9179):651–52.
83. Fanchin R, Harmas A, Benaoudia F, Lundkvist U, Olivennes F, Frydman R. Microbial flora of the cervix assessed at the time of embryo transfer adversely affects in vitro fertilization outcome. *Fertil Steril.* 1998;70(5):866–70.
84. Moore DE, Soules MR, Klein NA, Fujimoto VY, Agnew KJ, Eschenbach DA. Bacteria in the transfer catheter tip influence the live-birth rate after in vitro fertilization. *Fertil Steril.* 2000;74(6):1118–24.
85. Salim R, Ben-Shlomo I, Colodner R, Keness Y, Shalev E. Bacterial colonization of the uterine cervix and success rate in assisted reproduction: Results of a prospective survey. *Hum Reprod.* 2002;17(2):337–40.
86. Selman H, Mariani M, Barnocchi N, Mencacci A, Bistoni F, Arena S, et al. Examination of bacterial contamination at the time of embryo transfer, and its impact on the IVF/pregnancy outcome. *J Assist Reprod Genet.* 2007;24(9):395–99.

27

ARTIFICIAL GAMETES

Oocytes

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Introduction

Improvements in human-assisted reproductive techniques (ART) have been hindered by our lack of knowledge of the mechanisms regulating human oocyte development from formation through to maturation. Whilst there is an increasing understanding of these processes in rodent models, there is a need for tractable human model systems. The capacity to follow human oocyte development entirely *in vitro* would provide insights into the basic science of oogenesis, folliculogenesis, and meiosis and would lead to the development and improvement of ART. The production of *in vitro*-derived gametes, whether from immature gametes (primordial follicles) or from stem cells, has been described as “artificial gametes.” Apart from providing important models for research, if they are shown to be safe, they would reduce the need for donor eggs and sperm as well as provide more fertility preservation options for a wider group of people. This chapter will cover the progress in producing so-called “artificial oocytes” from a range of cell types and consider the technology of growing oocytes *in vitro* from the most immature stages to maturity.

Source of artificial oocytes

Formation of oocytes *in vivo* occurs during fetal life in the human ovary. Primordial germ cells (PGCs) migrate to the presumptive ovary where they become oogonia and upon entering meiosis become oocytes that are enclosed within somatic cells (granulosa cells) to form primordial follicles. Primordial follicles consist of an oocyte arrested at the dictyate stage of prophase 1 of meiosis enclosed within flattened somatic (granulosa) cells and form the non-proliferating pool of germ cells from which recruitment for growth will take place throughout a woman’s reproductive life [1]. The number of follicles formed, the rate at which they are utilized, and exposure to gonadotoxic substances are all factors that determine female fertility. For women who have a high-risk of premature ovarian insufficiency (POI), fertility preservation options such as cryopreservation of ovarian tissue for subsequent re-implantation have been developed [2, 3]. This technique has resulted in the birth of more than 130 babies [4], but reimplantation is not suitable for all patient groups. For women who have few oocytes remaining, alternative strategies such as making new oocytes from stem cells and developing them *in vitro* are being considered as future therapies.

The *in vitro* differentiation (IVD) of oocytes from stem cells has clear application for fertility preservation, and there has been rapid progress in this field particularly using mouse models. Stem cells have the potential to provide a source of new oocytes that could be utilized to achieve fertility in women who are infertile or have an exhausted ovarian reserve. Research has focused on obtaining artificial oocytes from pluripotent cells, either

embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Another potential source is from unipotent oogonial stem cells or germline stem cells [5, 6].

Stem cells are undifferentiated or differentiation-limited, self-renewing cells within a distinct niche. Pluripotent stem cells have the ability to differentiate into all the cells of a mammalian embryo and therefore have the potential to generate germ cells. Mammalian oogenesis *in vivo* is a tightly coordinated process which requires the transient switching on and off of regulatory genes and molecular processes, of which we still have limited knowledge (Figure 27.1).

Oocytes from pluripotent stem cells (ESCs and iPSCs)

The inner cell mass of the developing blastocyst forms the pluripotent epiblast cells which give rise to somatic tissues and germ cells. Derivation of two types of stem cell lines from the mouse epiblast has been achieved: (i) embryonic stem cells (ESCs) [7] and (ii) epiblast stem cells (EpiSCs) [8]. These cell lines can differentiate into somatic and germline lineages [8]. Embryonic stem cells from human blastocysts (hESCs) have been derived [9] and have been considered candidate progenitor cells for *in vitro* oogenesis [10].

Differentiating germ cells have been derived from mouse embryonic stem cells (mESCs) [11]. Isolation of these cells based on Oct4 and cKit expression showed a range of germ cell developmental stages (migratory primordial germ cells and post-migratory germ cells) (Figure 27.1). Oocyte-like cells (OLCs) were identified from cultured mESCs but these were not functional oocytes as they did not undergo meiosis [11]. These experiments highlighted that the formation of OLCs occurs independently of the process of meiosis as confirmed by experiments in mice on Stra8-deficient ovarian germ cells where oocytes are formed, but meiosis does not take place [12].

Identifying early germ cells within culture shows that the earliest stages of the complex pathway for germ cell development can be recapitulated *in vitro*. Before germ cell migration, germ cell fate is induced in the epiblast cells in mice via bone morphogenic protein 4 (BMP4) signalling from the surrounding soma [13]. Epiblast-like cells (EpiLCs) have been induced from mESCs with a gene expression profile consistent with pre-gastrulating epiblasts. BMP4 induced expression of Blimp1 in EpiLCs and led to upregulation of Nanos3, Dppa3, and Prdm14 associated with primordial germ cell specification and downregulation of somatic markers Hoxa1, Hoxb1, and Snai1. These changes occurred alongside epigenetic changes replicating *in vivo* differentiation of epiblast cells into primordial germ cells [14]. These results demonstrate successful differentiation of EpiLCs to primordial germ cell-like cells (PGCLCs) *in vitro* comparable to that occurring *in vivo*.

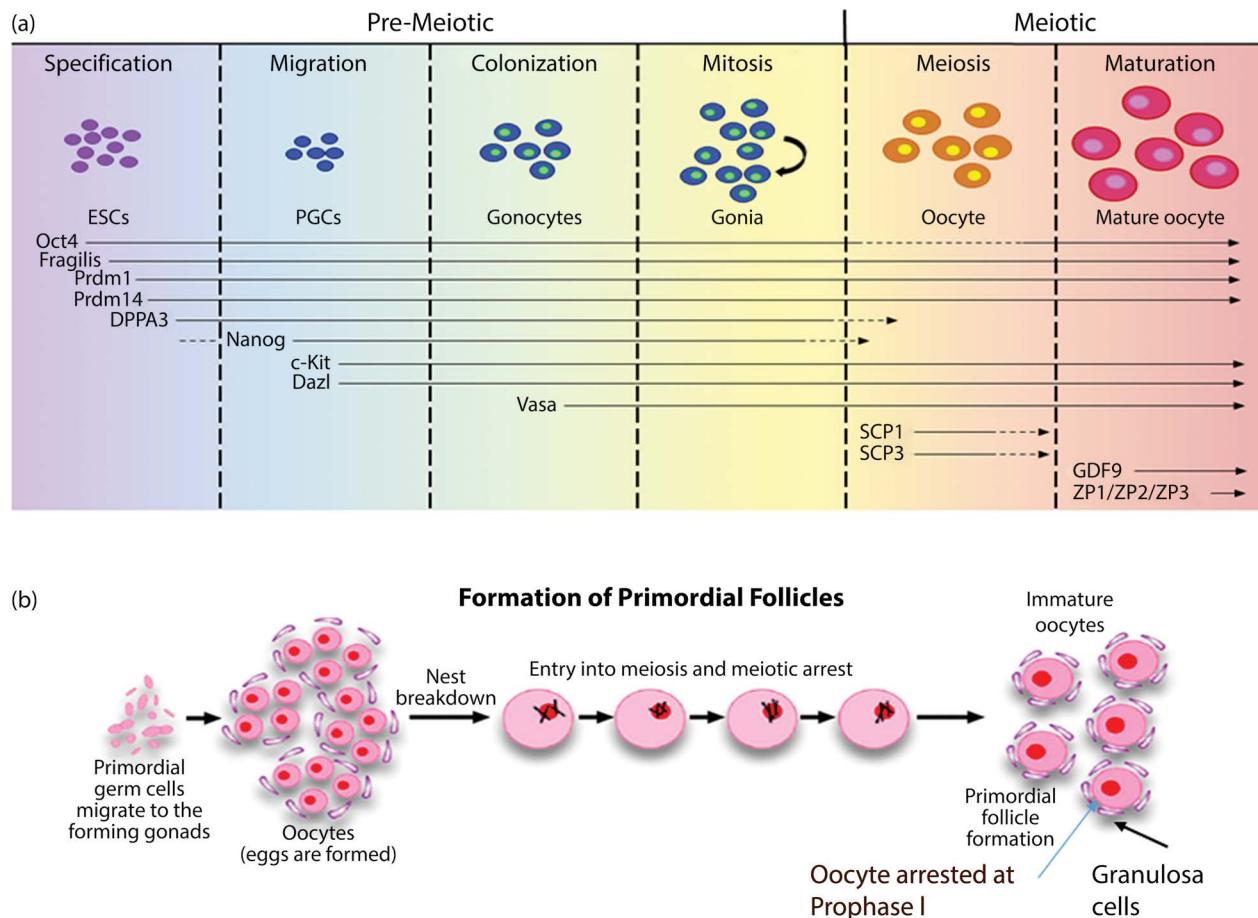


FIGURE 27.1 (a) Stages of germ cell formation/development and expression levels of germ cell markers. (b) The process of follicle formation from PGCs to oogonia entering meiosis to form oocytes that are arrested at prophase I of meiosis, surrounded by somatic cells to form primordial follicles. Abbreviations: ESC, embryonic stem cell; PGC, primordial germ cell.

BMP4-dependent differentiation of PGCLCs could be inhibited by Noggin (BMP4 antagonist), whereas another mesoderm promoting factor (Wnt3a) also induced PGCLCs in culture [15], illustrating the importance of somatic factors in *in vitro* differentiation of germ cells. PGCLCs derived from embryoid bodies (EB) differentiated into oocyte-like cells with expression of oocyte-specific genes (Fig, GDF9, ZP1, ZP2, and ZP3) and early meiotic marker (SCP3) when co-cultured with granulosa cells [16], similar results were observed when PGCLCs were co-cultured with Chinese hamster ovary cells [17]. However these results could not be replicated with granulosa cell conditioned medium [16], confirming the importance of cell–cell interactions with ovarian somatic cells (Figure 27.2).

When PGCLCs were combined with embryonic ovarian somatic cells and xenotransplanted to the ovarian bursa of immune-deficient recipient mice, oocyte-like cells enclosed within follicles were formed. The oocyte-like cells were capable of being matured and fertilized *in vitro* and embryos were produced. The resultant offspring were healthy, fertile, and showed normal imprinting patterns [18]. These studies demonstrated the potential of mESCs to undergo differentiation to all stages of oogenesis and subsequent embryonic development, and, as with *in vivo* oogenesis, interactions with surrounding somatic cells are essential for successful *in vitro* oogenesis (Figure 27.2).

Germ cell differentiation of human ESCs has been investigated and PGCLCs have been derived from hESCs with gene expression patterns similar to PGCs [19]. The differentiation of hESCs to germ cell precursors occurs spontaneously but it has been shown that the addition of BMP4 increases the rate of differentiation [20]. Several growth factors and feeder layers have been utilized to improve the differentiation rate cells with germline and meiotic markers have been obtained (reviewed by [21]). Follicle-like structures that express oocyte-specific markers (ZP1 and GDF-9) were formed in EBs derived from hESCs; however, a zona pellucida could not be detected in the presumptive oocyte [22]. More recently, hESCs have developed into oocyte-like structures [23], but meiosis has not been observed.

Whilst research using ESCs gives us insight into cell lineage development, the use of human ESCs clinically is fraught with practical difficulties and ethical concerns. A major concern is that derivation of oocytes from human ESCs for clinical application would be dependent on somatic cell nuclear transfer as the cells would not be biologically related to the recipient [24] and many ethical concerns surround this. Therefore, it is unlikely that derivation of gametes by this route would be applied clinically and a more likely route would be to utilize induced pluripotent stem cells (iPSCs) derived from adult cells, which overcomes the difficulties associated with hESCs [10].

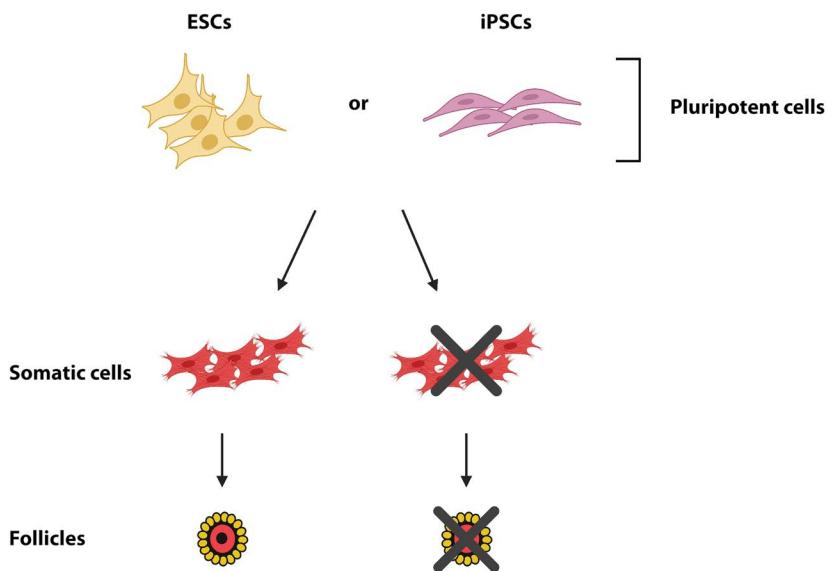


FIGURE 27.2 Illustrating the essential step of combining somatic cells with primordial germ cell-like cells to form follicles *in vitro*. If somatic cells are absent, no follicles will be formed. Abbreviations: ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells.

Oocytes from iPSCs

Methodology to dedifferentiate and induce pluripotency in adult cells was developed by Takahashi and Yamanaka and led to the generation of the first iPSC lines [25, 26]. Mouse fibroblasts under the expression of four crucial pluripotency genes—Oct3/4, Klf4, Sox2, and c-Myc—were induced to a pluripotent state and termed induced pluripotent cells (iPSCs) [26], and iPSCs have now been derived from several species including humans [25]. These cells have been used to regenerate several tissue types and are becoming clinically viable [27]. Given that iPSCs do not raise the same ethical concerns as ESCs they are more likely to provide a clinical option for artificial gametogenesis.

Using a tetraploid complementation assay, mouse iPSCs have demonstrated their ability to generate all cell types including germ cells [28, 29], similar to ESCs. Mouse iPSCs can be derived *in vitro* to EpiLCs and PGCLCs and have been combined with somatic cells to form a reconstituted ovary, after xenotransplantation oocytes have been generated (reviewed by [30]). Some of these oocytes are meiotically and developmentally competent, and offspring have been produced [18]. Following on from this, it has been demonstrated that competent oocytes can be derived from stem cells entirely *in vitro*, avoiding the need for a transplantation stage [31] (Figure 27.3). Hikabe et al. developed a multi-step system that supports *in vitro* differentiation, *in vitro* growth, and *in vitro* maturation to produce developmentally competent oocytes entirely *in vitro*.

Each stage of development is supported using medium containing a combination of factors specific for each stage [31]. Stage one supports the generation of PGCLCs from iPSCs or mESCs using media with a mixture of growth factors then combining the PGCLCs with embryonic ovarian cells to make an ovarian organoid which supports the formation of oocytes/follicles within 21 days of reaggregation [31]. Stage two supports follicle/oocyte growth *in vitro* in media containing BMP15, GDF-9, and FSH to produce fully grown oocyte–cumulus complexes that can be matured in stage three. Stage three utilizes

standard IVM protocols to mature these *in vitro*-derived oocytes. Some of the oocytes reached Metaphase II, were fertilized, and offspring were produced [31]. Whilst the offspring produced were healthy and epigenetically normal, the success rate was low, and many unhealthy oocytes were produced with less than 4% resulting in the formation of embryos [31]. A greater understanding of the factors regulating oocyte formation/early development is required, and recently a group of transcription factors that can produce oocyte-like cells *in vitro* from embryonic stem cells has been identified [32]. These findings represent major progress in defining the mechanisms required to produce good oocytes [33].

Clearly, the ability to derive oocytes from iPSCs entirely *in vitro* is a huge step, but this protocol [31] relied on using embryonic tissue as a source of somatic cells to support germ cell development. The use of embryonic/fetal tissue is not a viable option if these protocols are ever to be applied to humans and utilized clinically. Another major advance in this field has shown the development of ovarian somatic cell support from PSCs [34]. Under defined culture conditions, mESCs can be differentiated into fetal ovarian somatic cell-like cells (FOSLCs) [34], and these can be combined with PGCLCs derived from mESCs to form aggregates that support the formation of follicles with functional oocytes capable of being fertilized, developing embryos leading to healthy offspring [34] (Figure 27.3). The ability to form functional oocytes/follicles without the need to utilize embryonic somatic cells is a major advance and sets the scene for developing support cells from iPSCs derived from adult cells thus improving techniques for human and other species [30].

Induced pluripotent stem cells (iPSCs) from human cells (hiPSCs) have been developed to germ-like cells with post-meiotic cells being induced [35]. PGCLCs have been induced from human iPSCs [36, 37], and more recently PGCLCs and oogonia have been derived from hiPSCs and combined with human fetal-derived somatic cells to form follicle-like structures [38]. These developments bring us closer to human oocytes being derived entirely *in vitro* from hiPSCs.

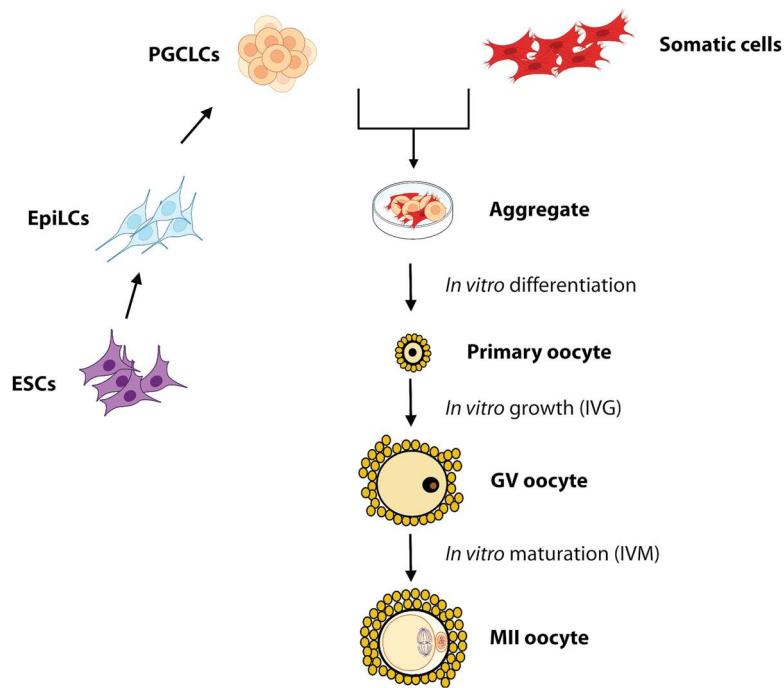


FIGURE 27.3 Steps to achieve complete *in vitro* formation of ovarian follicles from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). [31] used embryonic tissue to obtain somatic cells to support germ cell development, whereas [34] derived somatic support cells from pluripotent stem cells (PSCs) demonstrating the complete recapitulation of germ cell development *in vitro* forming competent oocytes capable of being fertilized and forming embryos. Abbreviations: ESCs, embryonic stem cells; EpiLCs, epiblast-like cells; PGCLCs, primordial germ cell-like cells; GV, germinal vesicle; MII, metaphase II.

There has been tremendous progress in the development of oocytes from mESCs and iPSCs in mice. With the birth of apparently healthy offspring, there is now proof of principle that artificial oocytes can be produced from mESCs and iPSCs. Human studies are making good progress but there is still much work required to determine the long-term safety of these methods and to translate it to human oocyte development.

Oocytes from somatic cell transformation

The hierarchical stem cell differentiation model has recently been challenged by the cell plasticity model, which describes that cells possess the ability to cross traditional lineage barriers [39]. The ability to manipulate a somatic cell to transdifferentiate (conversion of a differentiated cell of one lineage to a differentiated cell of another lineage without reinstating pluripotency) by direct reprogramming could also allow the *in vitro* generation of new oocytes. Skin-derived stem cells (SDSCs) isolated from neonatal mice have been shown to differentiate into PGCLCs *in vitro* [40, 41]. SDSC-derived PGCLCs underwent epigenetic changes similar to PGCs *in vivo* and activin A promoted PGCLC differentiation [41] similar to human ESCs. SDSCs also generated aggregates, morphologically similar to follicles, containing large cells with expression of oocyte-specific markers, when cultured alone and with ovarian cells derived from neonatal mice. The large oocyte-like cells expressed meiotic markers, but SCP3 showed discontinuous staining patterns, consistent with the cells' inability to progress through meiosis. SDSCs aggregated with neonatal ovarian somatic cells and transplanted to the kidney capsule of recipient mice generated oocyte-like cells in developing follicles

through to antral stages of development [40], suggesting SDSCs can differentiate and generate cells that have morphological similarities to oocytes, again confirming the separation of formation of oocyte structures from meiotic capacity [12].

Fetal porcine SDSCs have also demonstrated a potential for germ cell differentiation *in vitro*. PGCLCs derived from fetal porcine SDSCs express germ cell markers (Dppa3, Dazl, Vasa, and cKit) and also show imprint erasure [42]. Further development of these PGCLCs to OLCs in aggregates demonstrates the presence of a zona pellucida and expression of oocyte and meiotic markers. Rat pancreatic stem cells have also generated OLCs in culture with a structure similar to a zona pellucida, in aggregation with smaller cells, and share gene expression patterns with oocytes, with the presence of oocyte and meiotic markers expressed [43]. Human amniotic fluid stem cells have also been able to recapitulate this differentiation pathway *in vitro*, generating aggregates with large central cells (OLCs) surrounded by a zona pellucida structure and smaller cells which produced oestrogen during culture. Analysis of the OLCs showed the expression of oocyte-specific and meiotic markers and underwent parthenogenetic activation during prolonged culture [44, 45], consistent with previous reports from other cell types and species.

A recent study has shown that functional oocytes with genomic stability can be generated from adult mouse ovarian somatic granulosa cells [46]. Using a chemical reprogramming approach granulosa cells could be induced to form PSCs through reprogramming using crotonic acid. These gPSCs (granulosa pluripotent stem cells) acquired germline potential and could form PGCLCs which produced functional oocytes that could be fertilized and produced fertile offspring [46].

It is clear that some cells can be reprogrammed under certain conditions and can form morphological oocytes, but in most cases these do not enter meiosis. As emphasised earlier, the process of oocyte differentiation can be dissociated from meiosis [12], so if functional artificial oocytes are to be obtained then it will be essential to understand the connection between oocyte differentiation and entry into meiosis.

Oocytes from oogonial stem cells (OSCs)

In recent years there have been some exciting and controversial developments in female germ cell biology relating to an increasing body of evidence that shows oocytes may be formed by a rare population of putative germline stem cells that can be isolated from the adult ovary [5, 47–49]. These cells are proposed to be germ lineage-specific rather than being pluripotent cells but their contribution to the pool of oocytes is still unclear. This chapter will not deal with their potential physiological role and will only consider their potential utility *ex vivo*.

The isolation and identification of oocyte-producing germline stem cells, also referred to as oogonial stem cells (OSCs) from adult mammalian ovaries was reported in 2009 when putative germline stem cells were isolated from adult mouse ovaries [50]. Isolation of similar cells from adult human ovaries followed [51]. These cells have now been isolated from the ovaries of adult mice [50, 51], rats [52], and humans [51, 53–55].

The isolation of these cells has been based on magnetic or fluorescent (FACS) cell sorting, utilizing an antibody to a germ cell marker DEAD box polypeptide 4 (DDX4) [50, 51, 53]. The isolation process has led to controversy, as DDX4 is assumed to be localized internally rather than being expressed on the surface,

although there is evidence that DDX4 can be expressed on the cell surface [56]. Some groups have failed to isolate the cells using similar methodologies [57, 58], whereas others have isolated a population of cells which have a molecular signature which includes germ and stem cell markers [50, 51] in rats [52] and humans [51, 53–55]. In human ovarian tissue, this is a rare cell population, comprising 0.014% of the total cell population which can stably proliferate *in vitro* for months and spontaneously generate oocyte-like structures, as determined by morphology and gene expression [51], and will also form follicle-like structures *in vitro* when combined with fetal somatic cells [53].

Injection of fluorescently labelled mouse OSCs into recipient fertile and infertile mouse ovaries has generated GFP-positive oocytes within host somatic cells and these have been capable of ovulation, fertilization, and embryonic development [50, 51], and in some cases live young have been produced [50, 52]. Human OSCs (hOSCs) have also generated OLCs enclosed in host somatic cells, as assessed by morphology and expression of oocyte-specific markers after injection into adult human ovarian cortical tissue and xenotransplantation into an immune-deficient mouse for seven days [51, 59]. Putative OSCs isolated from adult human ovaries combined with human fetal ovarian-derived somatic cells (FODSCs) *in vitro*, form oocyte/follicle-like structures in 57% of the aggregates [53]. Figure 27.4 summarizes results to identify the oogenic potential of OSCs from human ovaries.

In addition to putative OSCs, another population of stem cells have been isolated from the adult human ovary that have characteristics of very small embryonic-like stem cells (VSELs) [60, 61]. These cells have been isolated and comprise a population of small cells of less than 5 microns that express germline and stem cell markers and appear to have oogenic potential [62]. It is thought

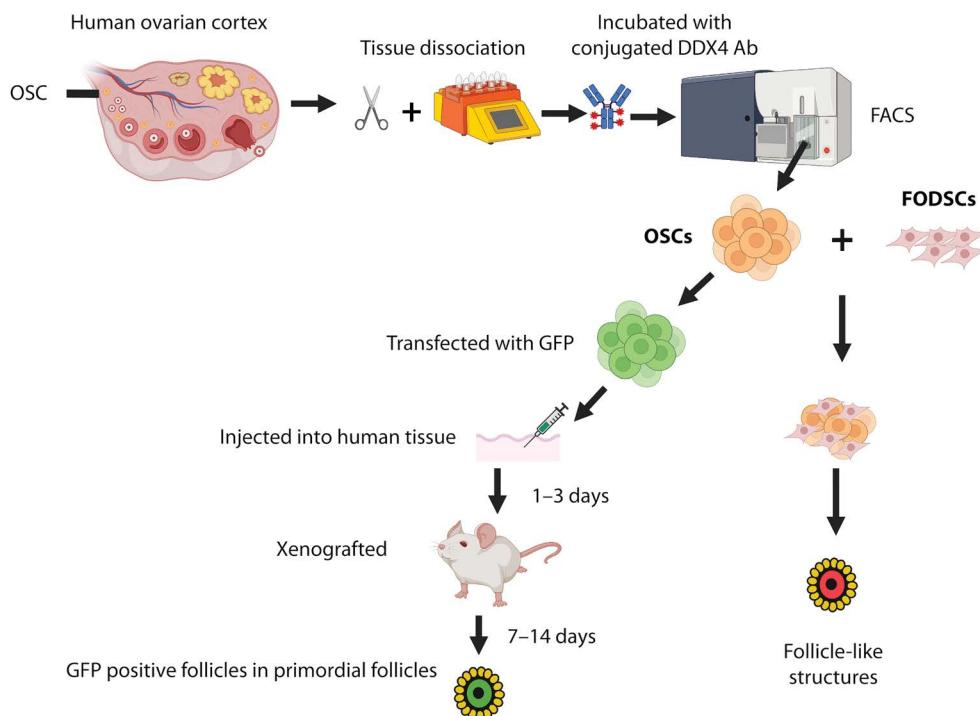


FIGURE 27.4 Oogonial stem cells (OSCs) isolated from adult human ovaries form oocyte structures within follicles if injected into human ovarian cortical tissue and xeno-transplanted to immune-deficient mice [51]. Oocyte/follicle structures can also be formed *in vitro* if OSCs are combined with human fetal ovarian-derived somatic cells (FODSCs) [53].

that these VSELs are precursors to OSCs [63], but there remains a great deal of work to be conducted to determine cell lineages in the adult human ovary.

Whilst identifying cells with apparent germline potential in the human ovary represents a major development, it has also led to a great deal of controversy. The methods used to isolate these cells still need to be clarified, but given the evidence from several groups they should be investigated further. These cells present an opportunity to learn more about germ cell development and the processes involved. The “oocyte-like” cells derived from each of the cell types discussed require somatic cell support of paracrine and junctional communication to form follicles and to support development into functional oocytes. Combining these “oocyte-like” cells with ovarian culture models may facilitate follicle formation and growth [64].

Supporting oocyte formation and growth *in vitro*

Obtaining viable oocytes by growing immature oocytes *in vitro* has been the subject of a great deal of research for more than 30 years. Whilst this is not the production of “artificial gametes,” the techniques to support *in vitro* oocyte growth are essential to support the development of a gamete being developed from any source. Complete growth *in vitro* from the most immature oocytes (primordial stages) with subsequent *in vitro* fertilization (IVF) and production of live young has only been achieved in mice [65, 66]. Early work on this two-step culture system resulted in only one live offspring being obtained, and this mouse had many abnormalities as an adult [65]. Following improvements in the technique and after alterations in the culture medium several mouse embryos and offspring have been obtained using oocytes that have been *in vitro* grown (IVG) combined with *in vitro* maturation (IVM) and IVF [66]. This work has provided proof of concept that complete oocyte development can be achieved *in vitro* and has facilitated the work on mouse ESCs and iPSCs described earlier. This has led to the development of culture systems that could be applied to other species, particularly human. Advances in culturing follicles from humans, non-human primates, and domestic species had been made; bringing the prospect of achieving an *in vitro* system that supports complete human oocyte development closer [64, 67].

In vitro growth systems

A defining feature of primordial follicles formed *in vivo* is that they enter a resting phase and must be activated to initiate growth (Figure 27.5). Activation and growth of primordial follicles is marked by the transformation of the flattened epithelial cells surrounding the oocyte into cuboidal cells which proliferate, forming a multilaminar structure in which the germ cell will develop. Normal follicle/oocyte development is critically dependent upon intercellular communication between the growing oocyte and the developing granulosa cells, therefore support and maintenance of these connections are essential [68]. During follicle development, the oocyte is held in meiotic arrest, but as it grows it must acquire the ability to resume meiosis (meiotic competence) and the ability to support fertilization and embryonic development (developmental competence). The development of culture conditions to support germ cell development is an enormous challenge, and an understanding of the physiological requirements of each component of the developing follicle is needed.

Initiation of primordial follicle growth

Primordial follicles represent the dormant store of follicles, and their activation is regulated via complex interactions of paracrine factors mediated by oocyte–somatic cell interactions, all of which are influenced by biomechanical forces [69]. Supporting this complex multi-layered process *in vitro* is technically challenging, but complete human oocyte development from primordial/unilaminar follicles to meiotic maturation has been achieved [70, 71]. All systems being developed to support human oocyte development start with either ovarian cortex or whole ovaries that have been removed for fertility preservation [64]. If ovarian cortex is being used then this tissue will contain mainly primordial follicles, whilst growing follicles can be isolated from whole ovaries.

Primordial follicles isolated from human ovarian tissue are not activated to grow *in vitro* [72], but the activation of human primordial follicles occurs spontaneously within ovarian cortex [73–76] and occurs in higher numbers over a shorter timeframe if the tissue has been mechanically loosened [70, 77]. Activated follicles can develop to multilaminar (secondary) stages within six days [77]. The density of stromal cells and tissue architecture are emerging as critical factors contributing to the regulation of activation of growth *in vitro*. The fragmentation of ovarian tissue that

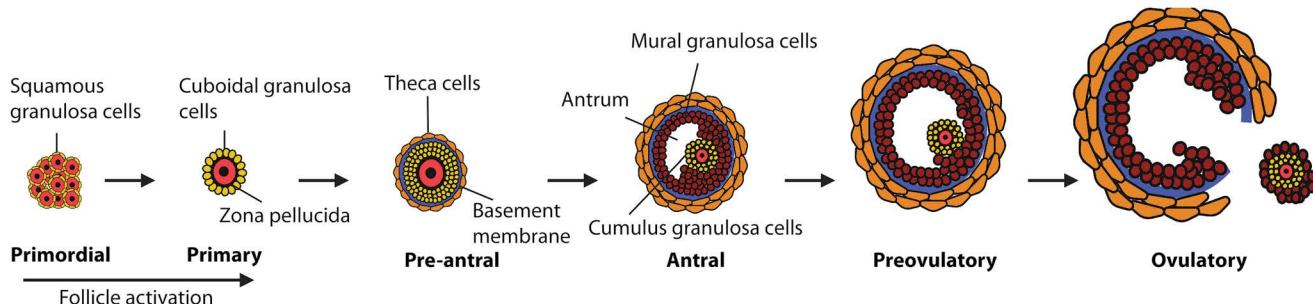


FIGURE 27.5 Stages of ovarian follicle development. Primordial follicles represent a pool of non-growing follicles which are continually initiated to grow throughout reproductive life. Once follicles are activated to grow (primary stage) granulosa cells proliferate to form multi-laminar structures (pre-antral) and then form a fluid-filled cavity (antral) which undergoes expansion to reach pre-ovulatory stages and the oocyte–cumulus complex being released at ovulation in response to luteinising hormone (LH) signalling.

occurs during the preparation of tissue into fragments of micro-cortex affects the Hippo signalling pathway which controls organ size through regulating cell proliferation and cell death [78]. In addition to the Hippo signalling pathway, a key cell signalling pathway involved in regulating primordial follicle activation is the phosphatidylinositol-3'-kinase (PI3K-AKT) pathway [79, 80].

IVG of preantral follicles

Once follicles have been initiated to grow within ovarian micro-cortex (Figure 27.6), they develop to the preantral/secondary stage but when they reach this stage the cortical environment that they are embedded within becomes inhibitory to further growth, resulting in a loss of follicle integrity and oocyte survival [77]. This inhibitory effect can be overcome by removing growing follicles from the micro-cortex and placing them in individual culture wells to limit the effect of follicle interactions [77].

Culture systems have been developed to support the growth of multilaminar (preantral) follicles that have been isolated as growing follicles from human ovarian cortex [81–85] or developed *in vitro* from primordial stages [70, 71, 77, 86]. Maintaining the structure of isolated human ovarian follicles is challenging as they can grow up to several millimetres. Tissue engineering principles have been applied to tackle this problem and several groups have encapsulated human preantral follicles within biomaterials such as alginate to support their structure and promote their growth *in vitro* [82, 84, 85]. In addition to alginate there has been development of a range of scaffolds to support human follicle growth *in vitro*. These include de-cellularized ovarian tissue [83, 87] and three-dimensional micro-porous scaffolds [88, 89].

A supporting matrix is not required to promote the development of isolated follicles and the multi-step culture system that has been developed for human follicles does not use them (Figure 27.6) [70]. Individual multilaminar follicles are placed within v-shaped micro-well plates with serum-free medium containing a low dose of FSH, Activin-A, and ascorbic acid [70] (Figure 27.6). Growth and differentiation of preantral follicles takes place within this system and three-dimensional architecture is maintained *in vitro* in human [77, 86, 90] and cow follicles [91], with antral formation occurring within 10 days (Figure 27.6).

Once antral cavities have formed, oocyte–granulosa cell complexes can be retrieved by applying gentle pressure to the follicle [70]. Complexes with complete cumulus and adherent mural

granulosa cells are then selected for further growth on membranes in step three of the multi-step system (Figure 27.6).

The aim of this stage is to promote oocyte growth given that oocyte size is an indicator of meiotic potential, and following this step oocytes of at least 100 microns can be obtained and selected for further maturation. Some IVG oocytes derived from the multi-step culture system undergo meiotic maturation following an IVM protocol with approximately 30% of oocytes that survive the entire culture period forming Metaphase II spindles [70]. Polar bodies formed by the IVGM oocytes are significantly larger than normal [70], but it is not known if this impacts developmental potential.

An important application of IVG would be to prepubertal girls who have few options for fertility preservation [92], but significant differences exist in the follicle population with age and stage of pubertal maturation [86]. Follicles derived from prepubertal girls grow at a different rate *in vitro* compared to those derived from adults, therefore culture systems developed for adult tissue may not be suitable for prepubertal girls. Adaptations and refinement for specific age groups and origin of oocytes will be required.

Final stages of growth and maturation

The end point of an *in vitro* system is to produce oocytes that can be fertilized and produce developmentally normal embryos. In order to achieve this, *in vitro*-grown human oocytes need to be matured *in vitro* to resume meiosis and reach Metaphase II (MII). The production of meiotically competent MII oocytes from human IVG follicles has been achieved [70, 93]. Whilst both systems supported oocyte growth to a diameter of >100 µm which could be selected for IVM, and oocytes reached Metaphase II, the polar bodies in the oocytes grown from the primordial stage were larger than expected [70]. More recent work utilizing a multi-step culture system over a prolonged period (nine weeks) has resulted in successful maturation of IVG oocytes to the MII stage following IVM, and all with normal-sized polar bodies [71]. Whether these IVG mature oocytes are developmentally competent remains to be assessed. Additionally, studies comparing IVG mature oocytes derived from fast or protracted culture systems are urgently needed to determine which one will provide the most adequate support for oocyte function, chromosome arrangement, epigenetic imprinting, and health.

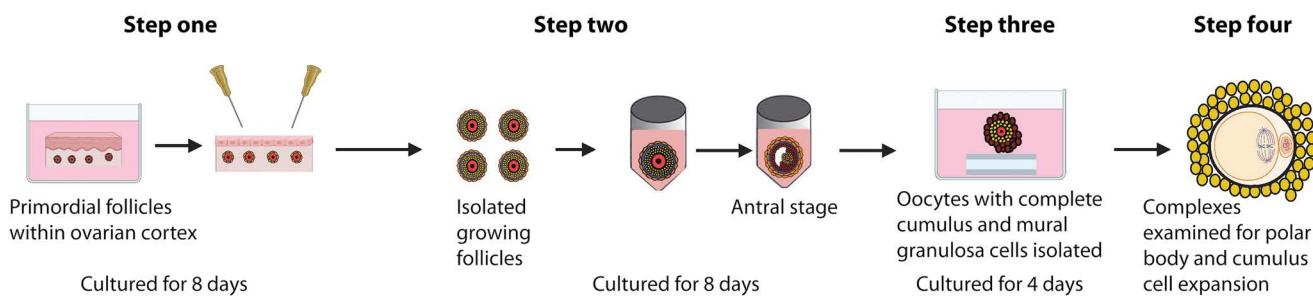


FIGURE 27.6 A multi-step culture system for human follicle/oocyte development [70]. Step one: Pieces of ovarian tissue containing primordial/unilaminar follicles are prepared for culture. Once follicles have reached multi-laminar stages, they can be mechanically isolated using needles. Step two: Isolated follicles are cultured individually from preantral to antral stages. Step three: Cumulus–oocyte complexes (COCs) are retrieved from the antral follicles and further cultured until oocyte diameter is >100 µm. Step four: COCs are placed within medium for *in vitro* maturation (IVM) and then examined for cumulus cell expansion (yellow), metaphase II spindle formation, and the presence of a polar body.

Whilst these studies provide proof of concept that complete IVG of human oocytes is possible, the clinical use of IVG culture systems is still limited by low MII rates, ambiguous fertilization capacities, and unknown epigenetic safety. As well as improvements in IVG systems there is also a need to utilize more precise IVM techniques. Advances in IVM techniques that involve a pre-maturation phase have been made [94, 95]. This technique helps to prevent spontaneous meiotic maturation that occurs *in vitro* whilst maintaining synchronization of oocyte nuclear and cytoplasmic maturation. This pre-maturation is carried out in the presence of C-type natriuretic peptide ("capacitation" step – CAPA), followed by conventional IVM (CAPA-IVM). There is now accumulating evidence that CAPA-IVM leads to increased oocyte maturation rates, enhanced embryo quality, and higher pregnancy rates [96–98]. Nevertheless, further refinement and optimization of IVM protocols are still required to (i) develop and validate a standardized, efficient and safe IVM system and (ii) improve the maturation rate and developmental potential of IVG-derived oocytes.

Conclusion

Improvements in ART require a greater understanding of the mechanisms regulating human oocyte development from formation through to maturation. The capacity to follow human oocyte development entirely *in vitro* would provide insights into the basic science of oogenesis, folliculogenesis, and meiosis, potentially leading to the development and improvement of ART. The generation of healthy progeny from stem cells in mice has brought new hope for restoring female fertility. Nevertheless, advances in the development of the human germline *in vitro* have been modest compared to mouse, and the genetic stability and functionality of the cells is still uncertain. Developing these techniques into mature, safe, and replicable processes following International Society for Stem Cell Research guidelines [99] is ongoing, but there is still a long way to go before artificial oocytes could be used clinically.

References

- Oktem O, Oktay K. The ovary: Anatomy and function throughout human life. *Ann N Y Acad Sci.* 2008;1127:1–9.
- Anderson RA, Baird DT. The development of ovarian tissue cryopreservation in Edinburgh: Translation from a rodent model through validation in a large mammal and then into clinical practice. *Acta Obstet Gynecol Scand.* 2019;98(5):545–9.
- Donnez J, Dolmans MM. Fertility preservation in women. *N Eng J Med.* 2017;377(17):1657–65.
- Lotz L, Dittrich R, Hoffmann I, Beckmann MW. Ovarian tissue transplantation: Experience from Germany and worldwide efficacy. *Clin Med Insights Reprod Health.* 2019;13:1179558119867357.
- Oqani RK, So S, Lee Y, Ko JJ, Kang E. Artificial oocyte: Development and potential application. *Cells.* 2022;11(7):1135.
- Sarma UC, Findlay JK, Hutt KJ. Oocytes from stem cells. *Best Pract Res Clin Obstet Gynaecol.* 2019;55:14–22.
- Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* 1981;292(5819):154–6.
- Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature.* 2007;448(7150):191–5.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998;282(5391):1145–7.
- Hendriks S, Dancet EA, van Pelt AM, Hamer G, Repping S. Artificial gametes: A systematic review of biological progress towards clinical application. *Hum Reprod Update.* 2015;21(3):285–96.
- Hübner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, et al. Derivation of oocytes from mouse embryonic stem cells. *Science.* 2003;300(5623):1251–6.
- Dokshin GA, Baltus AE, Eppig JJ, Page DC. Oocyte differentiation is genetically dissociable from meiosis in mice. *Nat Genet.* 2013;45(8):877–83.
- Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, et al. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev.* 1999;13(4):424–36.
- Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell.* 2011;146(4):519–32.
- Wei W, Qing T, Ye X, Liu H, Zhang D, Yang W, et al. Primordial germ cell specification from embryonic stem cells. *PLoS One.* 2008;3(12):e4013.
- Qing T, Shi Y, Qin H, Ye X, Wei W, Liu H, et al. Induction of oocyte-like cells from mouse embryonic stem cells by co-culture with ovarian granulosa cells. *Differentiation.* 2007;75(10):902–11.
- Eguizabal C, Shovlin TC, Durcova-Hills G, Surani A, McLaren A. Generation of primordial germ cells from pluripotent stem cells. *Differentiation.* 2009;78(2–3):116–23.
- Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from *in vitro* primordial germ cell-like cells in mice. *Science.* 2012;338(6109):971–5.
- Clark AT, Rodriguez RT, Bodnar MS, Abeyta MJ, Cedars MI, Turek PJ, et al. Human STELLAR, NANOG, and GDF3 genes are expressed in pluripotent cells and map to chromosome 12p13, a hotspot for teratocarcinoma. *Stem Cells.* 2004;22(2):169–79.
- Duggal G, Heindryckx B, Warrier S, O'Leary T, Van der Jeugt M, Lierman S, et al. Influence of activin A supplementation during human embryonic stem cell derivation on germ cell differentiation potential. *Stem Cells Dev.* 2013;22(23):3141–55.
- Yao C, Yao R, Luo H, Shuai L. Germline specification from pluripotent stem cells. *Stem Cell Res Ther.* 2022;13(1):74.
- Afshar A, Ruban L, Jones M, Afshar A, Fazeli A, Moore HD. In vitro post-meiotic germ cell development from human embryonic stem cells. *Hum Reprod.* 2009;24(12):3150–9.
- Jung D, Xiong J, Ye M, Qin X, Li L, Cheng S, et al. In vitro differentiation of human embryonic stem cells into ovarian follicle-like cells. *Nat Commun.* 2017;8:15680.
- Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, et al. Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell.* 2013;153(6):1228–38.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131(5):861–72.
- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126(4):663–76.
- Yamanaka S. Pluripotent stem cell-based cell therapy—promise and challenges. *Cell Stem Cell.* 2020;27(4):523–31.
- Boland MJ, Hazen JL, Nazor KL, Rodriguez AR, Gifford W, Martin G, et al. Adult mice generated from induced pluripotent stem cells. *Nature.* 2009;461(7260):91–4.
- Zhao XY, Li W, Lv Z, Liu L, Tong M, Hai T, et al. Viable fertile mice generated from fully pluripotent iPS cells derived from adult somatic cells. *Stem Cell Rev Rep.* 2010;6(3):390–7.
- Saitou M, Hayashi K. Mammalian *in vitro* gametogenesis. *Science.* 2021;374(6563):eaaz6830.
- Hikabe O, Hamazaki N, Nagamatsu G, Obata Y, Hirao Y, Hamada N, et al. Reconstitution *in vitro* of the entire cycle of the mouse female germ line. *Nature.* 2016;539(7628):299–303.
- Hamazaki N, Kyogoku H, Araki H, Miura F, Horikawa C, Hamada N, et al. Reconstitution of the oocyte transcriptional network with transcription factors. *Nature.* 2021;589(7841):264–9.

33. Schultz RM, Eppig JJ. Challenges to making an egg. *Nat Cell Biol.* 2021;23(1):9–10.
34. Yoshino T, Suzuki T, Nagamatsu G, Yabukami H, Ikegaya M, Kishima M, et al. Generation of ovarian follicles from mouse pluripotent stem cells. *Science.* 2021;373(6552) eabe0237.
35. Eguizabal C, Montserrat N, Vassena R, Barragan M, Garreta E, Garcia-Quevedo L, et al. Complete meiosis from human induced pluripotent stem cells. *Stem Cells.* 2011;29(8):1186–95.
36. Irie N, Weinberger L, Tang WW, Kobayashi T, Viukov S, Manor YS, et al. SOX17 is a critical specifier of human primordial germ cell fate. *Cell.* 2015;160(1-2):253–68.
37. Sasaki K, Yokobayashi S, Nakamura T, Okamoto I, Yabuta Y, Kurimoto K, et al. Robust in vitro induction of human germ cell fate from pluripotent stem cells. *Cell Stem Cell.* 2015;17(2):178–94.
38. Yang S, Liu Z, Wu S, Zou L, Cao Y, Xu H, et al. Meiosis resumption in human primordial germ cells from induced pluripotent stem cells by in vitro activation and reconstruction of ovarian nests. *Stem Cell Res Ther.* 2022;13(1):339.
39. Estrov Z. Stem cells and somatic cells: Reprogramming and plasticity. *Clin Lymphoma Myeloma.* 2009;9(Suppl 3):S319–28.
40. Dyce PW, Liu J, Tayade C, Kidder GM, Betts DH, Li J. In vitro and in vivo germ line potential of stem cells derived from newborn mouse skin. *PLoS One.* 2011;6(5):e20339.
41. Sun R, Sun YC, Ge W, Tan H, Cheng SF, Yin S, et al. The crucial role of Activin A on the formation of primordial germ cell-like cells from skin-derived stem cells in vitro. *Cell Cycle.* 2015;14(19):3016–29.
42. Linher K, Dyce P, Li J. Primordial germ cell-like cells differentiated in vitro from skin-derived stem cells. *PLoS One.* 2009;4(12):e8263.
43. Danner S, Kajahn J, Geismann C, Klink E, Kruse C. Derivation of oocyte-like cells from a clonal pancreatic stem cell line. *Mol Hum Reprod.* 2007;13(1):11–20.
44. Cheng X, Chen S, Yu X, Zheng P, Wang H. BMP15 gene is activated during human amniotic fluid stem cell differentiation into oocyte-like cells. *DNA Cell Biol.* 2012;31(7):1198–204.
45. Yu X, Wang N, Qiang R, Wan Q, Qin M, Chen S, et al. Human amniotic fluid stem cells possess the potential to differentiate into primordial follicle oocytes in vitro. *Biol Reprod.* 2014;90(4):73.
46. Tian C, Liu L, Ye X, Fu H, Sheng X, Wang L, et al. Functional oocytes derived from granulosa cells. *Cell Rep.* 2019;29(13):4256–67.e9.
47. Akahori T, Woods DC, Tilly JL. Female fertility preservation through stem cell-based ovarian tissue reconstitution in vitro and ovarian regeneration in vivo. *Clin Med Insights Reprod Health.* 2019;13:1179558119848007.
48. Martin JJ, Woods DC, Tilly JL. Implications and current limitations of oogenesis from female germline or oogonial stem cells in adult mammalian ovaries. *Cells.* 2019;8(2):93.
49. Telfer EE, Anderson RA. The existence and potential of germ-line stem cells in the adult mammalian ovary. *Climacteric.* 2019;22(1):22–6.
50. Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, et al. Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nature Cell Biology.* 2009;11(5):631–6.
51. White YA, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat Med.* 2012;18(3):413–21.
52. Zhou L, Wang L, Kang JX, Xie W, Li X, Wu C, et al. Production of fat-1 transgenic rats using a post-natal female germline stem cell line. *Mol Hum Reprod.* 2014;20(3):271–81.
53. Clarkson YL, McLaughlin M, Waterfall M, Dunlop CE, Skehel PA, Anderson RA, et al. Initial characterisation of adult human ovarian cell populations isolated by DDX4 expression and aldehyde dehydrogenase activity. *Sci Rep.* 2018;8(1):6953.
54. Ding X, Liu G, Xu B, Wu C, Hui N, Ni X, et al. Human GV oocytes generated by mitotically active germ cells obtained from follicular aspirates. *Sci Rep.* 2016;6:28218.
55. Silvestris E, Cafforio P, D'Oronzo S, Felici C, Silvestris F, Loverro G. In vitro differentiation of human oocyte-like cells from oogonial stem cells: Single-cell isolation and molecular characterization. *Hum Reprod.* 2018;33(3):464–73.
56. Clarkson YL, Weatherall E, Waterfall M, McLaughlin M, Lu H, Skehel PA, et al. Extracellular localisation of the C-terminus of DDX4 confirmed by immunocytochemistry and fluorescence-activated cell sorting. *Cells.* 2019;8(6):578.
57. Wagner M, Yoshihara M, Douagi I, Damdimopoulos A, Panula S, Petropoulos S, et al. Single-cell analysis of human ovarian cortex identifies distinct cell populations but no oogonial stem cells. *Nat Commun.* 2020;11(1):1147.
58. Zhang H, Panula S, Petropoulos S, Edsgård D, Busayavalasa K, Liu L, et al. Adult human and mouse ovaries lack DDX4-expressing functional oogonial stem cells. *Nat Med.* 2015;21(10):1116–8.
59. Grieve KM, McLaughlin M, Dunlop CE, Telfer EE, Anderson RA. The controversial existence and functional potential of oogonial stem cells. *Maturitas.* 2015;82(3):278–81.
60. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K, et al. Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary. *Stem Cells Dev.* 2011;20(8):1451–64.
61. Virant-Klun I, Zech N, Rozman P, Vogler A, Cvjeticanin B, Klemenc P, et al. Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes. *Differentiation.* 2008;76(8):843–56.
62. Virant-Klun I, Stimpfel M, Cvjeticanin B, Vrtacnik-Bokal E, Skutella T. Small SSEA-4-positive cells from human ovarian cell cultures: Related to embryonic stem cells and germinal lineage? *J Ovarian Res.* 2013;6:24.
63. Bhartiya D, Singh P, Sharma D, Kaushik A. Very small embryonic-like stem cells (VSELs) regenerate whereas mesenchymal stromal cells (MSCs) rejuvenate diseased reproductive tissues. *Stem Cell Rev Rep.* 2022;18(5):1718–27.
64. Telfer EE, Andersen CY. In vitro growth and maturation of primordial follicles and immature oocytes. *Fertil Steril.* 2021;115(5):1116–25.
65. Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod.* 1996;54(1):197–207.
66. O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod.* 2003;68(5):1682–6.
67. Telfer EE, Zelinski MB. Ovarian follicle culture: Advances and challenges for human and nonhuman primates. *Fertil Steril.* 2013;99(6):1523–33.
68. Li R, Albertini DF. The road to maturation: Somatic cell interaction and self-organization of the mammalian oocyte. *Nat Rev Mol Cell Biol.* 2013;14(3):141–52.
69. Shah JS, Sabouni R, Cayton Vaught KC, Owen CM, Albertini DF, Segars JH. Biomechanics and mechanical signaling in the ovary: A systematic review. *J Assist Reprod Genet.* 2018;35(7):1135–48.
70. McLaughlin M, Albertini DF, Wallace WHB, Anderson RA, Telfer EE. Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system. *Mol Hum Reprod.* 2018;24(3):135–42.
71. Xu F, Lawson MS, Bean Y, Ting AY, Pejovic T, De Geest K, et al. Matrix-free 3D culture supports human follicular development from the unilaminar to the antral stage in vitro yielding morphologically normal metaphase II oocytes. *Hum Reprod.* 2021;36(5):1326–38.
72. Abir R, Roizman P, Fisch B, Nitke S, Okon E, Orvieto R, et al. Pilot study of isolated early human follicles cultured in collagen gels for 24 hours. *Hum Reprod.* 1999;14(5):1299–301.
73. Garor R, Abir R, Erman A, Felz C, Nitke S, Fisch B. Effects of basic fibroblast growth factor on in vitro development of human ovarian primordial follicles. *Fertil Steril.* 2009;91(5 Suppl):1967–75.

74. Hovatta O, Silye R, Abir R, Krausz T, Winston RM. Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. *Hum Reprod.* 1997;12(5):1032–6.
75. Hovatta O, Wright C, Krausz T, Hardy K, Winston RM. Human primordial, primary and secondary ovarian follicles in long-term culture: Effect of partial isolation. *Hum Reprod.* 1999;14(10):2519–24.
76. Wright CS, Hovatta O, Margara R, Trew G, Winston RM, Franks S, et al. Effects of follicle-stimulating hormone and serum substitution on the in-vitro growth of human ovarian follicles. *Hum Reprod.* 1999;14(6):1555–62.
77. Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod.* 2008;23(5):1151–8.
78. Zhao B, Tumaneng K, Guan KL. The hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nat Cell Biol.* 2011;13(8):877–83.
79. Grosbois J, Demeestere I. Dynamics of PI3K and hippo signaling pathways during in vitro human follicle activation. *Hum Reprod.* 2018;33(9):1705–14.
80. Grosbois J, Devos M, Demeestere I. Implications of nonphysiological ovarian primordial follicle activation for fertility preservation. *Endocr Rev.* 2020;41(6):bnaa020.
81. Gruhn JR, Kristensen SG, Andersen CY, Hoffmann ER. In vitro maturation and culture of human oocytes. *Methods Mol Biol.* 2018;1818:23–30.
82. Jones ASK, Shikanov A. Follicle development as an orchestrated signaling network in a 3D organoid. *J Biol Eng.* 2019;13:2.
83. Pors SE, Ramløse M, Nikiforov D, Lundsgaard K, Cheng J, Andersen CY, et al. Initial steps in reconstruction of the human ovary: Survival of pre-antral stage follicles in a decellularized human ovarian scaffold. *Hum Reprod.* 2019;34(8):1523–35.
84. Shea LD, Woodruff TK, Shikanov A. Bioengineering the ovarian follicle microenvironment. *Annu Rev Biomed Eng.* 2014;16:29–52.
85. Xu M, Barrett SL, West-Farrell E, Kondapalli LA, Kiesewetter SE, Shea LD, et al. In vitro grown human ovarian follicles from cancer patients support oocyte growth. *Hum Reprod.* 2009;24(10):2531–40.
86. Anderson RA, McLaughlin M, Wallace WH, Albertini DF, Telfer EE. The immature human ovary shows loss of abnormal follicles and increasing follicle developmental competence through childhood and adolescence. *Hum Reprod.* 2014;29(1):97–106.
87. Laronda MM, Jakus AE, Whelan KA, Wertheim JA, Shah RN, Woodruff TK. Initiation of puberty in mice following decellularized ovary transplant. *Biomaterials.* 2015;50:20–9.
88. Laronda MM, Rutz AL, Xiao S, Whelan KA, Duncan FE, Roth EW, et al. A bioprosthetic ovary created using 3D printed micro-porous scaffolds restores ovarian function in sterilized mice. *Nat Commun.* 2017;8:15261.
89. Liverani L, Raffel N, Fattah A, Preis A, Hoffmann I, Boccaccini AR, et al. Electrospun patterned porous scaffolds for the support of ovarian follicles growth: A feasibility study. *Sci Rep.* 2019;9(1):1150.
90. McLaughlin M, Kinnell HL, Anderson RA, Telfer EE. Inhibition of phosphatase and tensin homologue (PTEN) in human ovary in vitro results in increased activation of primordial follicles but compromises development of growing follicles. *Mol Hum Reprod.* 2014;20(8):736–44.
91. McLaughlin M, Patrizio P, Kayisli U, Luk J, Thomson TC, Anderson RA, et al. mTOR kinase inhibition results in oocyte loss characterized by empty follicles in human ovarian cortical strips cultured in vitro. *Fertil Steril.* 2011;96(5):1154–9.e1.
92. Corkum KS, Rhee DS, Wafford QE, Demeestere I, Dasgupta R, Baertschiger R, et al. Fertility and hormone preservation and restoration for female children and adolescents receiving gonadotoxic cancer treatments: A systematic review. *J Pediatr Surg.* 2019;54(11):2200–9.
93. Xiao S, Zhang J, Romero MM, Smith KN, Shea LD, Woodruff TK. In vitro follicle growth supports human oocyte meiotic maturation. *Sci Rep.* 2015;5:17323.
94. De Vos M, Grynberg M, Ho TM, Yuan Y, Albertini DF, Gilchrist RB. Perspectives on the development and future of oocyte IVM in clinical practice. *J Assist Reprod Genet.* 2021;38(6):1265–80.
95. Richani D, Gilchrist RB. Approaches to oocyte meiotic arrest in vitro and impact on oocyte developmental competence. *Biol Reprod.* 2022;106(2):243–52.
96. Sanchez F, Le AH, Ho VNA, Romero S, Van Ranst H, De Vos M, et al. Biphasic in vitro maturation (CAPA-IVM) specifically improves the developmental capacity of oocytes from small antral follicles. *J Assist Reprod Genet.* 2019;36(10):2135–44.
97. Sánchez F, Lolicato F, Romero S, De Vos M, Van Ranst H, Verheyen G, et al. An improved IVM method for cumulus-oocyte complexes from small follicles in polycystic ovary syndrome patients enhances oocyte competence and embryo yield. *Hum Reprod.* 2017;32(10):2056–68.
98. Vuong LN, Le AH, Ho VNA, Pham TD, Sanchez F, Romero S, et al. Live births after oocyte in vitro maturation with a prematuration step in women with polycystic ovary syndrome. *J Assist Reprod Genet.* 2020;37(2):347–57.
99. Lovell-Badge R, Anthony E, Barker RA, Bubela T, Brivanlou AH, Carpenter M, et al. ISSCR guidelines for stem cell research and clinical translation: The 2021 update. *Stem Cell Reports.* 2021;16(6):1398–408.

28

HOW MICROFLUIDICS AND MICROFABRICATION WILL IMPROVE DIAGNOSIS AND TREATMENT IN HUMAN ART

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Introduction

Microfluidics is the science of manipulating small volumes of fluid typically in the range of microlitres to picolitres [1, 2]. Microfluidic components and platforms are being applied to many biomedical applications, by leveraging the precise levels of environmental control, cellular manipulation, and automation that these systems provide [3–6]. The term “lab-on-a-chip” is often applied to microfluidic platforms, which is due to the truly multidisciplinary nature of microfluidics, requiring equal contributions from fields such as biology, chemistry, physics, and computer software development, which are made possible thanks to the appropriation of traditional skills in microfabrication and the advent of new microfabrication methods from modern manufacturing [7–10]. Microfluidics arose initially with the development of inkjet printers in 1951, which harnessed the Hagen–Poiseuille equation, 1840, which was used to describe the characteristics of laminar flow of fluid through a tube [11]. Some decades later, in 1979, the merging of microfabrication with analytical methods resulted in the creation of the field of microfluidics [12]. Fabrication of devices using soft lithography for malleable and lightweight structures was adopted shortly thereafter in the early 1990s. Since then, microfluidic platforms have been used in numerous research and industrial applications within biotechnology, such as for point-of-care diagnostics (lateral flow immunoassays), tissue engineering and modelling (organ-on-a-chip), nanoparticle synthesis, drug discovery, and on-chip cellular analysis [13–16]. Compared to conventional approaches, these microfluidic platforms enable a higher quantity and quality of data to be gathered at a speed and price that is unmatched. Microfluidic devices also afford researchers excellent visualization of live cellular and physical reactions which are particularly valuable for the study of cells.

The modern assisted reproduction technology (ART) clinic consists of a myriad of highly regulated micro-environments with optimized layers of quality control and assurance to minimize external and internal stressors imposed on gametes and embryos throughout fertilization, embryo culture, and cryopreservation. These processes are all subject to the skill of the embryologist and successful operation of equipment. Microfluidics and microfabrication, in recent years, have attracted considerable attention for the many applications in which microlitre or nanolitre volumes of fluid could apply to the modern IVF laboratory or clinic. In the context of fertility and assisted reproduction, microfluidics has made several contributions, most notably in the areas of infertility diagnostics, gamete processing and analysis, embryo culture, on-chip fertilization, cryopreservation, and reproductive organ modelling (Figure 28.1) [1, 17, 18]. Microfluidics allows researchers to selectively mimic the geometry and environmental conditions present within reproductive systems, presenting opportunities for biomimetic emulations of *in vivo* processes

such as temperature, chemical, and physical environment. This has proven extremely useful for applications such as the assessment of oocyte and sperm quality and dynamics [17, 19, 20], and sperm selection for IVF, from both neat and frozen semen as well as testicular tissue from surgical sperm retrieval operations [17, 21, 22]. These platforms may employ “active” or “passive” approaches which apply either external forces (such as heat, current, or flow) or physical phenomena derived from a controlled geometry at the microscale. Microfluidics also offer precise control over heat transfer, which is an essential requirement for cryopreservation technologies [23] and has proven useful in sperm selection approaches [24]. Furthermore, paper-based microfluidic technologies offering simple, low-cost, and rapid diagnostic platforms are already available as pregnancy tests and are commonly employed outside ART [25].

More recently, microfluidics has also been applied in the modelling of complex tissue and organ micro-environments for reproductive science. These models can recapitulate the organ environments and endocrine signalling present naturally and may be able to provide reliable models for studying reproductive and whole-body health, *in vitro* drug screening, toxicity testing, and tissue transplantation [26–28]. Where 2D models lack the physiological relevance and three-dimensional (3D) architecture within tissue-tissue and multi-organ interactions microfabrication and microfluidics have been extensively utilized to develop better cell culture platforms than the existing conventional *in vitro* models [29]. However, despite impressive advances in the application of microfluidics for ART, translation of these technologies into clinical practice has been limited. Since the landmark first-generation work in lab-on-a-chip systems by Terry et al. in 1979 [12] and later by Manz et al. in 1990 [30], most systems are not yet realized as commercial products for research-grade instrumentation outside of specialist laboratories [31]. There are, however, notable exceptions, which this chapter will cover, illustrating a potential paradigm shift in laboratory processes and research for ART.

Here we consider how microfluidics, and more recently microfabrication, can be used for the analysis and diagnosis of sperm, and to enhance ART procedures including ICSI, *in vitro* maturation, embryo culture, and cryopreservation.

Microfluidics for semen analysis and diagnostics

The future of personalized infertility treatments will be bolstered by reliable and accurate diagnostics prior to clinical ART. In male infertility, semen analysis is the cornerstone of clinical diagnostics due to its relative simplicity and non-invasive nature; however, men often avoid voluntary proactive clinical assessment due to social stigma, leaving women to bear the burden of infertility in the initial stages [32]. Furthermore, notable biological variation

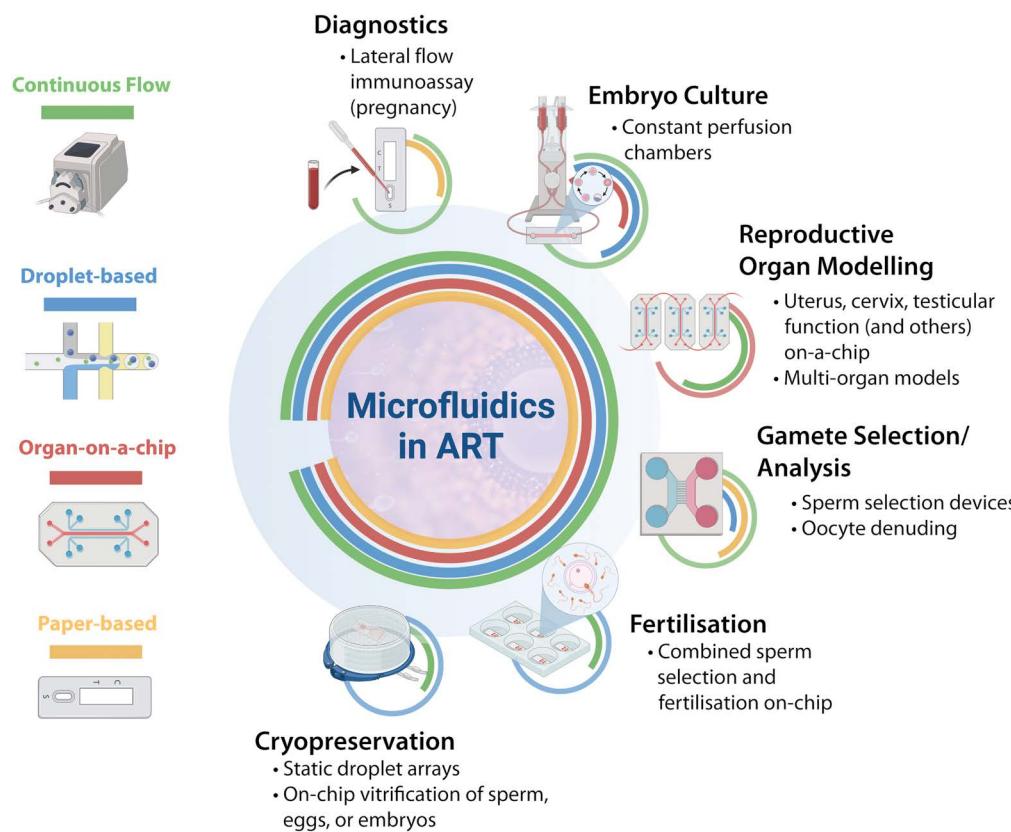


FIGURE 28.1 Summary of the applications to date of microfluidics in assisted reproductive technologies.

occurs between semen samples regarding basic semen parameters, thus semen analysis requires a robust, repeatable approach to standardize analysis by minimizing operator variability, while providing precise and accessible metrics indicative of patient fertility status [33]. Conventional clinical approaches to semen analysis have marginally improved with advancing technology, utilizing microfabricated counting chambers [34]; computer-aided sperm analysis (CASA) [35]; and assays for sperm viability [36], morphology, and DNA integrity [37]. These methods are nevertheless limited by factors such as poor standardization, high complexity, high cost, user variability, and extended processing times.

Concerted efforts to provide accessibility and de-stigmatization of male infertility diagnostics has seen several commercially viable systems developed for semen analysis in recent years. Traditional microfluidic devices, with miniaturized geometry and physics, consist of fabricated microchannels using materials such as polydimethylsiloxane (PDMS) and glass, enabling modular systems comprised of a series of chambers and valves, capable of incorporating multiple basic semen analysis techniques into a single device [38]. This can facilitate these devices to provide standardized metrics attempted by many computer-aided semen analysis CASA platforms. These proposed devices have the potential to reduce hands-on time and operator variability. Current CASA systems can assess sperm concentration, motility, kinematics, morphology, and vitality, which could theoretically be integrated with pH, viscosity, DNA fragmentation, and biomarker analysis using microfluidics. Alternatively, paper-based microfluidic approaches, which function by passively wicking

fluids via capillary action through paper that has selectively patterned hydrophobic boundaries [18], are specifically accessible and affordable formats for performing diagnostics.

Basic semen analysis

There have been several recent microfluidic methods of assessing sperm concentration, motility, and vitality which vary in both practicality and user-friendliness. Traditional microfluidic platforms based on sperm migration have shown promise for raw semen motility and concentration analysis (Figure 28.2a) [39] using parallel microchannels to separate motile sperm from immotile sperm and debris, and by measuring pellet size from each channel, concentration and motility is comparable to counting chambers. Fluorescently labelled sperm in microchannels have also been used with comparable results to CASA for total and progressive motility assessment, indicating sub-fertility based on WHO parameters [36, 40].

A commercial, at-home, paper-based rapid test such as the SpermCheck® Fertility device (SpermCheck, Fairfield, OH, US) provides a basic result indicating whether sperm concentration is normal, low, or very low using immunodiagnostic colorimetric signals (Figure 28.2b) [41]. Fertell (Genosis Ltd, Boston, US), another immunodiagnostic test used a swim-up chamber connected to a nitrocellulose strip trapping sperm labelled with anti-CD59 colloidal gold conjugate progressively motile sperm, indicating if progressive motility is over 10 million sperm per mL (Figure 28.2c) [42]. The Men's Rapid Fertility Test (LabCorp OnDemand, Burlington, VT, US) is another at-home test kit, using a compact, low-speed centrifuge and disposable microfluidic

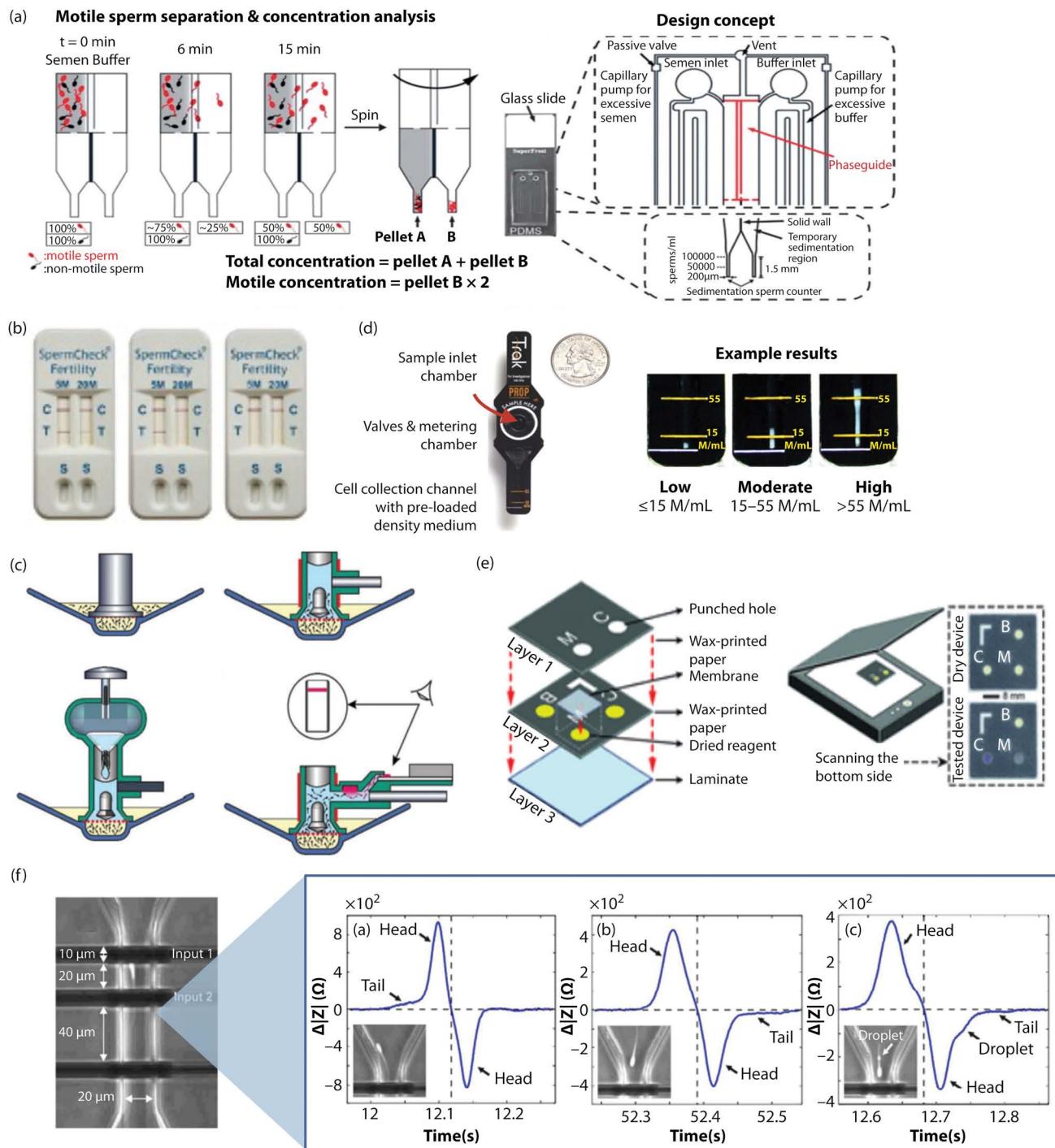


FIGURE 28.2 Microfluidic semen analysis examples showing a device (a) using parallel microchannels for motile sperm separation and assessing concentration based on pellet size; (b) the SpermCheck® Fertility device for sperm concentration assessment; (c) the Fertell immunodiagnostic test for sperm motility assessment; (d) the Men's Rapid Fertility Test for sperm concentration assessment; (e) an experimental paper-microfluidic device for sperm vitality assessment; (f) sperm morphology assessment using impedance. ([a] From [39] with permission; [b] from [41] with permission; [c] from [42] with permission; [d] from [43] with permission; [e] from [13] with permission; [f] from [45] with permission.)

chips, which funnel sperm into a microchannel column whereby column height indicates sperm concentration categorized into three broad ranges (Figure 28.2d) [43].

At-home tests for sperm motility, integrated with cloud-based analyses and mobile health strategies provide the ability for users

to take control of their treatment and receive medical advice without stepping foot into a clinic. CASA-based, at-home systems using microchamber slides such as the Yo Male Fertility Sperm Test (Mira, Pleasanton, CA, US) and the smartphone-based Sperm Test Kit (ExSeed Health, London, UK) aim to provide users with

digestible and simplified information by comparing sperm motility and concentration to reference values, and providing personalized advice from a cloud-based platform. CASA-based systems also have great potential for automating and standardizing basic semen analysis in the future, yet global adoption is currently hampered by cost, complexity, and standardization issues with calibration as a key factor which needs to be addressed.

Experimental microfluidic platforms for the analysis of sperm motility and concentration have been proposed using an easy-to-use paper-based approach to quantifying live and motile concentrations of sperm as well as sperm motility using colorimetric reaction of tetrazolium to purple formazan by an enzyme found in metabolically active sperm called diaphorase flavoprotein enzyme (Figure 28.2e) [13]. Paper-based microfluidics have also been applied to analysing DNA integrity of sperm using ion concentration polymerization (ICP) effects on nanoporous membranes on paper with a strong correlation to clinically performed flow-cytometry-based sperm chromatin structure assay (SCSA) [44]. Electrically integrated microfluidic chips have been developed to determine sperm concentration and morphologically normal sperm using sperm impedance cytometry and separating sperm from collateral cells and other sperm with large cytoplasmic droplets using dielectrophoretic sorting and counting (Figure 28.2f) [45]. This concept was explored using a planar platinum electrode pair between a microchannel with flowing sperm, which identified and counted sperm in a heterogenous sample using electrical impedance via a homemade impedance analyser [46]. Furthermore, another group showed an accurate differentiation of sperm with normal morphology versus those with cytoplasmic droplets using this technology [45].

Liquid biopsies

Microfluidics has also garnered attention recently in the field of liquid biopsies, providing non-invasive testing of disease states with high accuracy and actionable results. This complete control over fluid volumes and flow rates allows for high-resolution cell and particle separation within these platforms as well as enabling other parameters such as temperature, geometry, and mechanical stimuli [38]. Paper microfluidics, for example, has become widely used in point-of-care diagnostics as lateral flow immunoassays to rapidly test for Covid-19. The most common targets of this research and technology have been circulating tumour DNA (ctDNA), circulating tumour cells (CTCs), and small extracellular vesicles (S-EVs or exosomes) [47, 48]. In the context of infertility, S-EVs have been proposed as novel targets for diagnostics and point-of-need application, with extensive biomarker candidates within seminal plasma S-EVs being identified and proposed [49–53]. These observations have been complemented by an ever-growing body of evidence correlating S-EVs with many important physiological functions, pathophysiological states, and ubiquitous intercellular communication owing to their valuable cargo of proteins, lipids, metabolites, functional messenger RNA (mRNA) and microRNA (miRNA), and double-stranded DNA (dsDNA) [48, 54–58]. Seminal plasma is composed of secretions from the testes, epididymis, seminal vesicles, prostate, and bulbourethral glands, which provides a highly diverse cohort of S-EVs, free-floating proteins, nucleic acids, minerals, and other vesicles [59]. Isolating S-EVs however, due to their size, presents unique challenges and requires complex and laborious methods to isolate in pure populations.

The current gold standard for isolating S-EVs is differential ultracentrifugation, which requires high starting volumes

(millilitres), large complex equipment, and dedicated operators, and leads to significant loss of S-EVs during processing [48, 60]. Microfluidics, however, has been shown to effectively isolate S-EVs from microlitre starting volumes with high specificity and purity. Microfluidic approaches appropriate conventional methods such as immunoaffinity capture (using antibodies conjugated on nanobeads or surfaces to bind specifically to target exosomes) and filtration, but also employ methods unique to microfluidic geometry and physics such as acoustic isolation (using acoustic field radiation waves to focus and isolate particles with different size, density and compressibility) [61, 62] and nanowire trapping (trapping of S-EVs on surface-bound nanowires acting as a filter for S-EVs while allowing larger particles to pass through) [63]. Microfluidic devices can also incorporate multiple methods of S-EV isolation to improve both capture or detection efficiency as well as purity of target S-EVs, increasing the accuracy of measurements and conclusions made thereafter. Most microfluidic S-EV isolation platforms are purpose-built for cancer diagnostics with a blood-based or urine-based liquid biopsy approach and are designed for point-of-care applications. Applying microfluidic S-EV isolation and detection to infertility diagnostics and liquid biopsies would be better served using a point-of-need approach to direct and personalize treatments in ART, such as what type of insemination (IVF or ICSI) would lead to the greatest of successful live births based on the expression of biomarkers detected [53].

Microfluidic technology can enable new methods of analysing sperm quality as well as simplifying this process to provide accessible options for patients and clinicians. The unique advantage microfluidics offers is the ability to integrate multiple procedural steps within a single automated platform, highly relevant to semen analysis and liquid biopsies of an easily obtained fluid such as seminal plasma. Furthermore, with innovative development and validation, this technology can enable portability and cost-effectiveness in diagnostics and remove the need for large and expensive technology. This technology has applications in other biofluids obtained during ART such as ovarian follicular fluid and blood, and could open a new avenue of actionable diagnostics prior to, during, and after clinical infertility treatment.

Microfluidics for sperm selection

Sperm selection forms a core facet of ART that can considerably influence embryo development, miscarriage rates, and live birth rates [64–67]. In particular, the use of sperm with DNA fragmentation in IUI, IVF, or ICSI can negatively impact ART outcomes. However, the most commonly employed method of sperm selection, density gradient centrifugation (DGC) and swim-up (SU), remain largely unchanged and concerns around their safety and efficacy for ART have been reported with increasing frequency [68, 69]. Both DGC and SU have the potential to induce sperm DNA fragmentation through the production of reactive oxygen species (ROS) and iatrogenic damage as a result of centrifugation [70, 71]. In the female reproductive tract, a stringent series of selection mechanisms will filter all but a minute percentage of the starting population of ejaculated sperm [72, 73]. These natural mechanisms that have evolved in nature are able to discern the quality of sperm, yet are still poorly understood. The presumption that sperm are able to traverse the female reproductive tract to the oviduct are fecund is plausible but not certain. However, understanding the mechanisms that select sperm *in vivo* will clarify the properties of these sperm and inform the next generation

of sperm selection technologies, and may improve treatment outcomes when compared to the conventional methods widely used today [73, 74]. Additionally, the requirements for sperm selection can vary depending on the ART being performed, and this should be considered during the development of alternative sperm selection technologies.

Microfluidic sperm selection technology has progressed since the first peer-reviewed publication in 2003 [18, 72], although very few attempts have resulted in commercial products. Attempts at microfluidic sperm selection can be categorized into active and passive methods. Passive microfluidic devices rely upon their inherent geometry to manipulate fluids and the cells and compounds they may contain. Passive microfluidic devices typically select sperm by leveraging their motility and behaviour in confined microchannel environments [17, 21, 75–78]. These systems often make use of hydrostatic pressure (gravitational pressure from a column of fluid) and capillary forces (fluid movement in narrow micro or nanochannels) to forego the use of pumping systems to push fluids. This simplifies devices and makes them more accessible to researchers unfamiliar with microfluidics. Cho and Schuster [76] used such a system to select motile sperm capable of crossing laminar flow streams into fresh media, leaving behind dead and non-motile cells [76, 79] (Figure 28.3a). This device was reportedly able to select sperm with 97% motility and improved morphology ($9.5 \pm 1.1\%$ normal forms prior to sorting to $22.4 \pm 3.3\%$ normal forms after sorting). Later the same group went on to test clinically infertile samples and the device's ability to select sperm with improved DNA integrity, achieving a DNA fragmentation index (DFI) of less than 1% [80]. The treatable population for this device (like many existing ARTs) is limited to those with motile sperm cells and may not be applicable to those with low thresholds of motility. Asghar, Velasco [75] proposed a microfluidic chip consisting of a microchannel ending in a polycarbonate membrane (8 μm) (Figure 28.3b). This device processes neat semen samples, and achieved a separation of 85% and normal morphology averaging 30% [75]. While this device did display lower ROS than conventional SU methods, the use of a polycarbonate membrane may pose issues in clinical applications considering the potential for cell aggregates and tissue debris causing membrane blockage. The devices exhibited saturation at 30 minutes of operation, about half the time of a conventional DGC, but was limited in its throughput considering the use of a single channel. A similar design is shown in Figure 28.3c [81], which used a space-constrained model for sperm racing. Interestingly, the testing of both mouse and human sperm cells revealed a motility exhaustion of 30 and 60 minutes, respectively. Again, this study was benchmarked against SU methods but not DGC. However, over 30 minutes of sorting showed a 1.9-fold and 1.3-fold increase in velocity and motility, respectively, from raw samples [78]. Both devices have been commercialized, with the device by Asghar et al. receiving greater success due to its simplicity and robustness, and leading to the development of the Zymöt Fertility sperm selection device (Zymöt Fertility, Gaithersburg, MD, US) (Figure 28.3d).

While many microfluidic attempts at sperm selection suffer from low throughput, Nosrati and colleagues [21] developed a system of more than 500 radial microfluidic channels to process a large number of cells quickly. This device performed a one-step sperm purification and selection by processing 1 mL of raw semen, guiding sperm through boundary-following behaviour into a viscous media reservoir in 20 minutes (Figure 28.3e). This device also used a viscoelastic media, similar to the viscosity of

the mucous secretions of the oviducts [82]. This process achieved an 89% increase in sperm vitality and 80% improvement in DFI in clinically infertile samples. The group then went a step further and discovered that sperm able to follow the boundary of varying degrees of corners (without losing the wall) presented higher DNA integrity than normal straight-swimming cells [17]. Although the exact biological mechanism for this behaviour is not understood, turning sperm exhibited more than 50% better DNA integrity than straight-swimming sperm. To go further still, Yazadan Parast and colleagues appropriated the same concept to provide a 3D network of more than 560 micro-channels within a familiar syringe format [83]. This "sperm syringe" was able to recover 41% of sperm from diluted semen samples in under 15 minutes, and provided a considerable improvement in DNA integrity and morphology (Figure 28.3f). These studies demonstrate how controlled microfluidic geometries coupled with a detailed understanding of sperm kinematic behaviour may benefit ART. There is potential for this process to be improved using computer vision and improved media such as with the inclusion of AI or the use of molecular makers for sperm function [80, 83].

A wider goal of microfluidic devices is to parallelize or integrate several ART functions into one platform, thus conducting sperm selection, oocyte trapping, fertilization, and embryo culture on a single device. One example of this approach is a microfluidic device utilizing chemotaxis to guide mouse sperm towards oocytes through four perpendicular channels [84]. Sperm motility increased from 60% to 96% (at 15 minutes) when first measured at the inlet then near to the central wall, although motility did decline over time. However, the use of one interconnected fluid network with no fluid boundaries meant that several media exchange steps were necessary. This limits the clinical viability of this chip as it does little to alleviate the manual handling of media exchange, although it does remove the need for centrifugation to isolate sperm prior to use in insemination. Several other studies have developed microfluidic technologies for the investigation of chemotaxis and sperm but without the necessary ports for sperm selection, and are therefore purely investigative tools [24, 85].

Active microfluidics involves the use of external forces such as thermal, acoustic, or electromagnetic forces to influence fluids and reactions. Active microfluidic devices for ART have typically employed electrophoresis, thermotaxis, or light-induced dielectrophoresis [45, 86–88]. Thermotaxis is an established method of long-range sperm guidance [89]. The fallopian tube itself exhibits a thermal gradient of approximately 1.4°C, warming as the sperm travels in the direction of the oocyte. Several attempts have been made to introduce temperature as a means of sperm guidance; one such attempt by Li et al. investigated the thermotactic responses of motile sperm diverting into reservoirs adjacent to a primary channel after a constriction of the channel, mimicking the uterotubal junction (where *in vivo* thermotaxis begins) [87]. The channel shown in Figure 28.3g, uses an interfacial valve-closing mechanism to trap sperm once they have moved into warmer areas. Thermotactic responses were observed in 5.7%–10.6% of the motile sperm over four temperature ranges. While this is an indication that sperms have undergone (induced) capacitation and are thermally responsive, it is not a direct indicator of sperm fecundity or DNA integrity. Therefore, coupled with other techniques, such as passive wall guidance or chemotaxis, sperm fecundity may become more apparent. It is still not fully understood whether thermally responsive sperm alone are able to improve reproductive outcomes. While few studies have investigated the combinational effects of sperm selection mechanisms

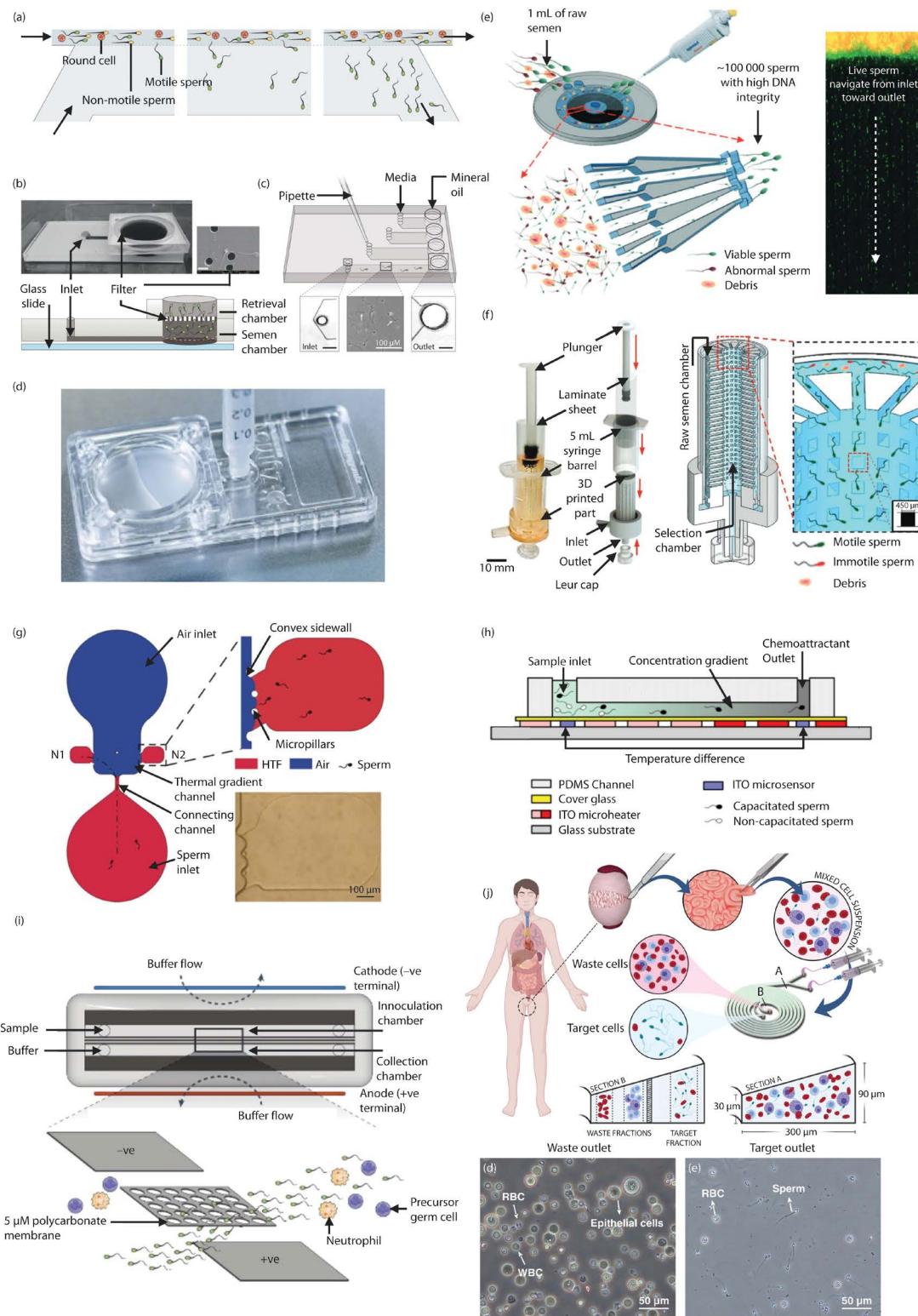


FIGURE 28.3 Microfluidic sperm selection platforms for use in treatment. (a) Simple channel for motile sperm selection against laminar flow streams; (b) polycarbonate membrane integrated with a microchannel to select motile sperm; (c) space-constrained model for sperm racing; (d) Zymöt sperm selection device using a membrane barrier to select motile sperm; (e) radial channel with viscous media to select sperm with boundary-following ability; (f) sperm syringe for motile sperm collected; (g) thermotactic intervalve sperm selection platform; (h) chemotaxis and thermotaxis platform to select motile sperm; (i) electric field selection of sperm across a polycarbonate membrane; (j) a microchannel spiral using inertial microfluidics to isolate sperm from surgical testicular tissue collections . ([a] From [18] with permission; [b] from [75] with permission; [c] from [81] with permission; [d] courtesy of ZymötR Fertility; [e] from [21] with permission; [f] from [83] with permission; [g] from [87] with permission; [h] from [24] with permission; [i] from [18] with permission.)

such as motility, chemotaxis, and thermotaxis, there are emerging trends in more recent studies that take this into consideration. Ko et al. designed a microfluidic device for the chemotaxis and thermotaxis assays of mouse sperm [24]. Their study demonstrates that the combination of these two selection mechanisms elicited a greater response in mouse sperm than when each method was used individually (Figure 28.3h). An alternative to chemotaxis that has made the transition into industry (with mixed success), is the use of electrophoresis, which has been used to select healthy sperm based on a combination of their size and charge [86]. This method uses an applied electric field to attract sperm across a polycarbonate membrane. The transition from one side of the membrane to another facilitates a media exchange from semen to fresh sperm media (Figure 28.3i). The use of this membrane excluded debris, leukocytes, and immature germ cells. This study reports that electrophoretic separation resulted in significantly improved vitality, motility, and DNA integrity compared with the initial sample, with more negatively charged sperm possessing high DNA integrity, although the exact reason why is not fully understood [90]. Using a fundamentally different approach to sort sperm may prove effective, but to date, active methods such as electrophoresis have yet to be validated clinically [91, 92].

Another niche within clinical sperm selection that often goes overlooked is the isolation and selection of surgically retrieved sperm. Microdissection and aspiration of testicular tissues typically entails several hours of manual sperm recovery [93]. Sperm recovery rates are low, and processing is prone to human error. Microfluidics (such as spirals) can preserve the vitality and DNA of the sperm by limiting their time *in vitro* and their exposure to ROS, digestive enzymes, cellular debris, and other contaminants [94]. Previously discussed forms of microfluidic sperm selection which leverage sperm motility are ineffective for non-motile surgically recovered sperm. As a result, several studies have appropriated various forms of inertial microfluidics, which makes use of fluid pumps, to leverage the unique morphology and size of sperm to filter out debris and concentrate sperm within a dramatically improved timeframe [22, 95, 96]. Across multiple studies, Son and colleagues demonstrated the novel application of inertial microfluidics to separate non-motile sperm from microbeads, red blood cells (RBCs), and white blood cells (WBCs) [97–99]. While promising, these studies worked with idealized cell suspensions largely unrepresentative of the triturated tissue and performed sperm separation from each cell type in isolation rather than a complex mixture. Recently, Vasilescu et al. used a similar spiral microchannel device fabricated by 3D printing that recovers sperm from heterogeneous cell suspensions of sperm, WBCs, RBCs, muscle epithelial cells, microparticles, and leukemic cancer cells (Figure 28.3j) [22]. Sperm were isolated within five minutes and, very importantly, were shown to have no detrimental impact on sperm viability, morphology, or DNA integrity.

Thus, microfluidic sperm selection has the potential to select higher-quality sperm with less error and greater standardization than conventional methods. While studies are limited, there is some preliminary evidence that suggests microfluidic sperm sorting does improve reproductive outcomes, including ongoing pregnancy rates in IUI, higher quality embryo generation, and improved chances of euploid conceptus [100–102]. Continued clinical application of microfluidic sperm selection will determine if this technology results in repeatable improvements in ART outcomes and for which aetiologies these new devices provide the most benefit.

Modelling reproductive organs

Developing and using translatable models of human reproduction has served as a consistent barrier to implementing safe molecular and pharmaceutical interventions for treating infertility. Microfabrication and microfluidics enable development of complex, 3D culture platforms with many benefits over conventional 2D culture [29]. These micro-engineered physiological models, appropriately termed organ-on-a-chip systems (OOCs), are more effective at mimicking the *in vivo* 3D multicellular architecture and micro-environment of the specific organ or tissue [103, 104]. OOCs can simulate blood flow with pump-controlled physiological fluid flow, enabling nutrient and gas perfusion along with mechanical stress cues [16]. OOCs technology has been applied to modelling both the female [28] and male reproductive systems with promising results.

Testes-on-a-chip (seminiferous tubules)

Multiple testes-on-a-chip models have been developed with varying goals in humans [105, 106], primates [106], and mice [107, 108]. A multi-organ human model was developed to study the interaction between a testicular organoid system and liver equivalent to observe natural and drug-induced tissue interactions (Figure 28.4a) [105]. This group observed testosterone and inhibin B production by the testicular organoids as well as observable steroid metabolism by the liver spheroids and germ cell loss when adding a chemotherapeutic drug (cyclophosphamide). A simple perfusion device enabled culturing, and studying of prepubertal primate seminiferous tubules was developed; tissue integrity, cell morphology, and viability was assessed under both hormonal stimulation and non-stimulation conditions [106]. Dulbecco's modified Eagles medium (DMEM) (Thermo Fisher, Waltham, MA, US) supplemented with 10% fetal bovine serum (FBS) was perfused into the device with FSH (0.5 IU mL⁻¹), hCG (0.5 IU mL⁻¹), and marmoset serum (0.4%) for non-stimulation conditions, and FSH (5 IU mL⁻¹), hCG (5 IU mL⁻¹), and marmoset serum (4%) for stimulation conditions. In mice, a bioreactor model for culturing testicular tissue in a purpose-built OOCs simulated *in vivo*-like conditions by creating a device consisting of a porous membrane separating cultured tissue from slowly flowed medium at 0.05 µL/min (Figure 28.4b) [107]. This device could maintain spermatogenesis and testosterone production in response to luteinizing hormone for six months, and produced functional sperm to generate healthy offspring using round spermatid injection (ROSI) and ICSI [107]. This same device was made pumpless using hydrostatic pressure for continuous infusion of α-minimum essential medium (α-MEM) supplemented with 40 mg mL⁻¹ bovine serum albumin (BSA) media [109] and altered further for improved visualization and monitoring of testes tissue during culture (Figure 28.4c) [108].

Such devices have considerable potential in both research and clinical applications, particularly as bioreactors to create usable sperm for use in ART. Creating robust and personalized platforms for culturing stem cells into usable sperm can be used to treat non-obstructive azoospermic patients and enable autologous gamete treatment options.

Oviduct-on-a-chip and uterus-on-a-chip

Monolayer culture of oviduct epithelial cells is notoriously difficult due to the rapid transformation of cuboidal columnar oviduct epithelial cells into flattened cells along with loss of beating cilia and reduced secretory function of these cells [110–112].

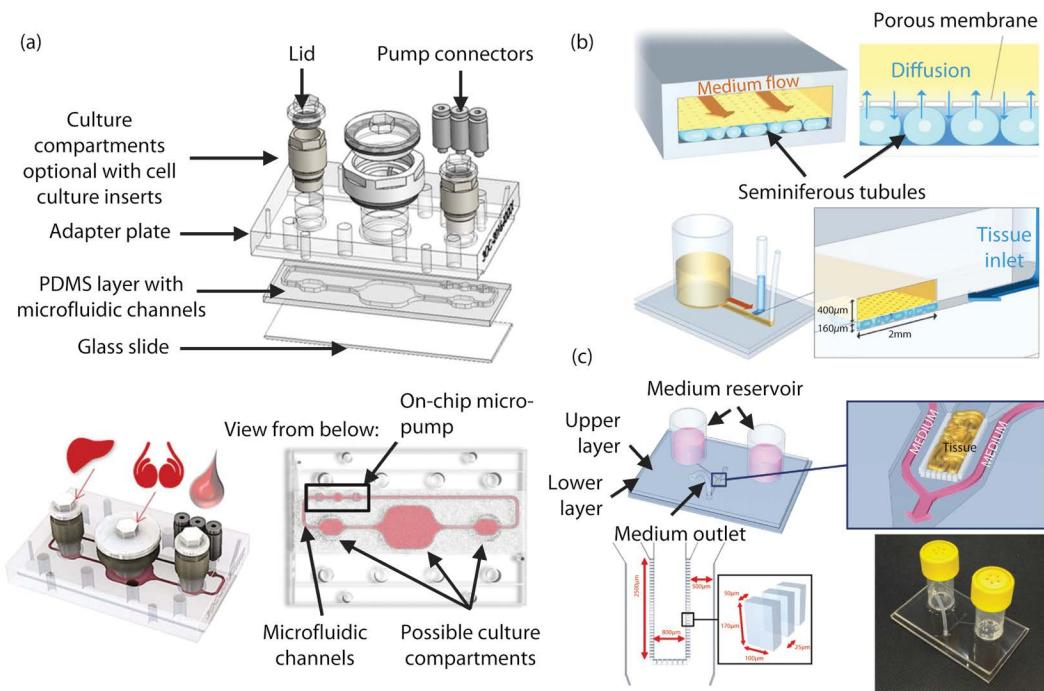


FIGURE 28.4 Male reproduction models showing (a) testicular tissue and liver equivalent model; (b) testicular tissue bioreactor to grow sperm from seminiferous tubules; (c) a pumpless diffusive perfusion version of this seminiferous tubule platform. ([a] From [105] with permission; [b] from [107] with permission; [c] from [108] with permission.)

Thus a bovine oviduct-on-a-chip was developed from a primary culture of bovine oviduct epithelium and was successful in maintaining beating cilia and secretory function of these cells (Figure 28.5a) [110]. This device was then used to facilitate sperm capacitation and IVF of bovine oocytes with reduced polyspermy when compared to conventional bovine IVF. Subsequently, this system was proposed as a mediator of improved fertilization in bovine models.

Recreating the uterine lining within a microfluidic OOC has been attempted using surgically excised human perivascular stroma and endothelial cells (Figure 28.5b) [113]. This system simulated temporal hormone changes during an idealized 28-day menstrual cycle and an enabled differentiation of stroma into functional decidual cells based on both morphology and prolactin production. Another “uterus-on-a-chip” model was developed using mice tissue which replicated some *in vivo* uterine functions to a greater extent, including implantation and embryo development within a physiologically favourable micro-environment [114]. Their device consisted of a co-culture of embryos and endometrial cells on either side of the porous membrane, allowing the diffusion of soluble factors as well as interaction of the embryo with the underlying cells through the membrane. When comparing embryo development in conventional petri dish culture versus their model, the uterus-on-a-chip model provided significantly higher morula and blastocyst rates [114]. This study provides an innovative approach to embryo culture and studying embryo development in an *in vivo*-like system. Mizuno et al. fabricated a uterus-on-a-chip platform mimicking the physicochemical features by co-culturing human endometrial cells with human zygotes [115]. Their microfluidic device improved blastocyst rates and overall embryo quality when compared to conventional microdrop culture.

Chang et al. developed a perfused 3D-uterus-on-a-chip to facilitate the co-culture of embryos with endometrial stromal cells in a dynamic manner to provide mild mechanical simulation (Figure 28.5c) [116]. The device also improved blastocyst rate by providing uterus-like conditions for the timely development of embryos and is thus proposed as an *in vivo*-like option for *in vitro* embryo culture. Another device was developed integrating both human uterine endometrial cells and ovarian follicular cells to simulate bidirectional endocrine crosstalk between the uterus and ovaries [117]. This device, in concert with a reliable reproductive toxicity marker, SERPINB2, was used to predict reproductive toxicity of specific chemicals introduced to the system and proposed as a substitute for animal models in testing these responses.

The biomimetic nature of reproductive organ-on-a-chip models provides a unique opportunity for simulating *in vivo* conditions for optimal fertilization and pre-implantation embryo development. Simulating these conditions by creating autologous oviduct epithelium and endometrial co-culture can support these important events with molecular and micro-environment interactions which could improve outcomes of fertilization and culture when compared to conventional culture systems. The clinical translation of these concepts is not a reality currently, however these models can be used to improve lab approaches to closer resemble the *in vivo* micro-environment of the oviduct and receptive uterus.

Entire female reproductive system on a chip

A female reproductive system-on-a-chip was developed using a single modular system with multiple docks for selected tissue and cell types to be cultured [28]. This chip provides a model capable of functional simulation of the 28-day menstrual cycle

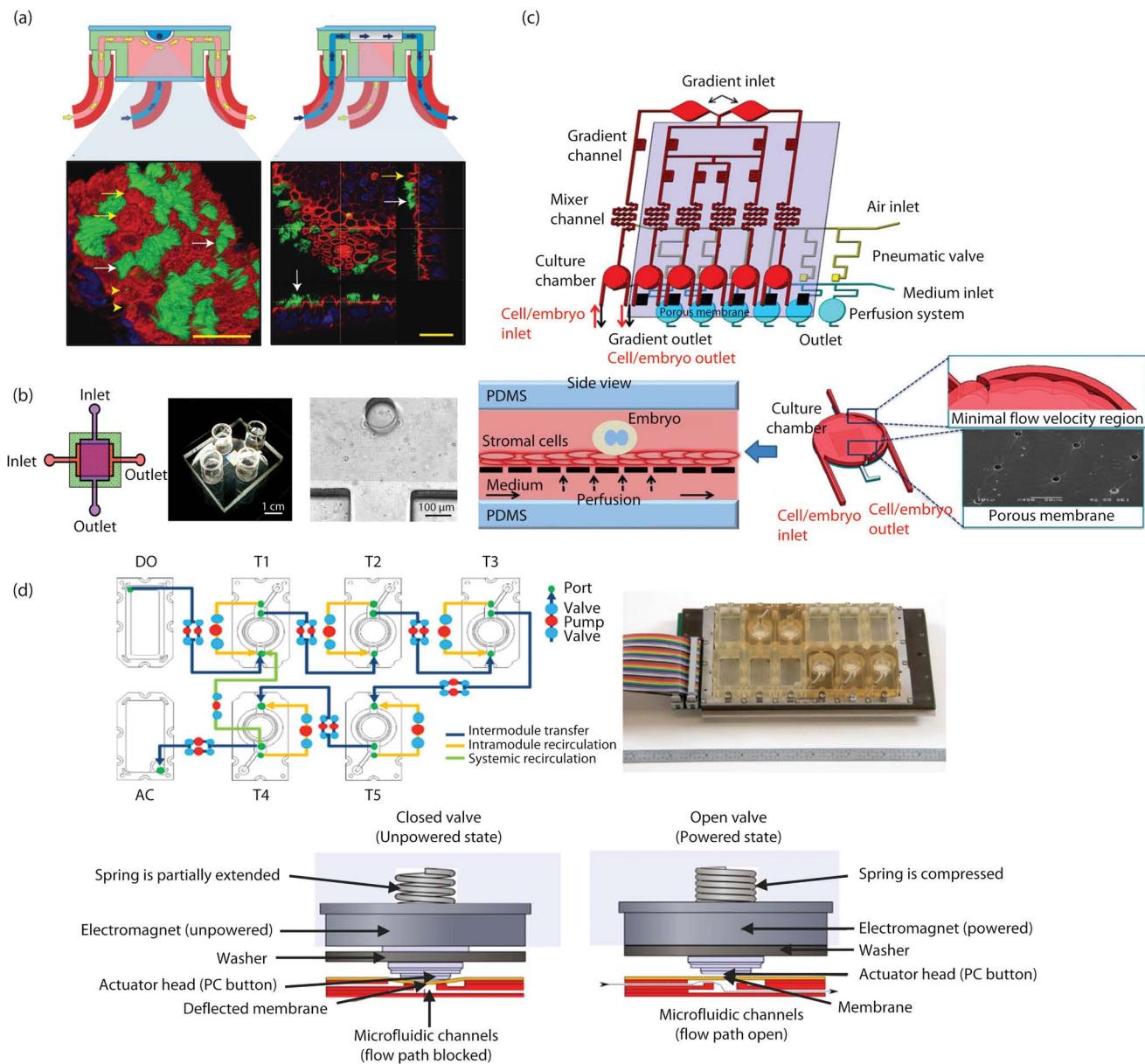


FIGURE 28.5 Female reproductive modelling: (a) bovine oviduct model; (b) human uterine lining model; (c) 3D-uterus-on-a-chip for embryo culture; (d) entire female reproductive system model. ([a] From [110] with permission; [b] from [113] under Creative Commons licence; [c] from [116] with permission; [d] from [28] under Creative Commons licence.)

including the culture, maturation, and differentiation of murine ovarian follicles (Figure 28.5d) [28]. Follicles were first perfused with growth medium containing 50% αMEM Glutamax and 50% F-12 Glutamax supplemented with 3 mg mL⁻¹ bovine serum albumin, 0.5 mg mL⁻¹ bovine fetuin, 5 mg mL⁻¹ insulin, 5 mg mL⁻¹ transferrin, and 5 mg mL⁻¹ selenium then on day 0, stimulated with maturation media 1.5 IU mL⁻¹ hCG, 10 ng mL⁻¹ epidermal growth factor, and 10 mIU mL⁻¹ FSH. These follicles successfully produced mature oocytes suitable for fertilization. This device was later integrated with organ modules for oviducts, uterus, cervix, and liver under continuous flow of media. This work presents a tool capable of mimicking micro-physiological interactions between the female reproductive tract and peripheral organs,

providing a unique method of studying pharmacodynamics and biological processes.

Although this field is in its infancy, further development in microfabrication technology and cell culture scaffolds can broaden the applications of OOCs in medicine and biology. OOCs are cost-effective, reproducible, scalable, and provide high throughput and precise information, which can reduce the dependency on conventional *in vivo* models [118]. With further advancements and incorporation of multiple organ-specific models with each other, OOCs represent novel platforms for screening drugs and toxins which may affect reproductive health, and help to identify new therapeutics by addressing a wide range of biological problems [119, 120]. Furthermore, incorporating

human-induced pluripotent stem cells (hiPSCs) could enable the development of patient-specific organ models, leading to individualized body-on-a-chip models to foster personalized medicine approaches to infertility.

Microfluidics for embryo culture

Development of the human pre-implantation embryo *in vivo* is highly dynamic from several perspectives. The embryo itself undergoes changes in its morphology and structure as it differentiates. It undergoes changes in its gene expression profile, all while undergoing dramatic changes in its metabolic functions. In parallel, the female tract provides different environments (nutritional, gaseous, pH, and signalling molecules) as the embryo progresses through the oviduct to the uterus, with the uterine environment providing an increasingly more complex milieu to support the development of the embryo post-compaction [121]. Furthermore, the embryo is in constant movement due to both ciliary and muscular activity of the female tract. All of this is in stark contrast to the way in which we have attempted to culture the pre-implantation embryo in the laboratory, i.e. in a drop of medium on a polystyrene culture dish/microwell (although sequential media were developed to accommodate physiological nutrient gradients). Gardner proposed in 1994 that in order to optimize embryo development and viability in the laboratory a dynamic/perfusion methodology could be applied [122] (see chapter by Gardner). In order to achieve these aims, several groups embarked on developing perfusion systems, initially using conventional peristaltic pumps with relatively high flow rates of 30 to 38 µL per minute [123] and subsequently using microfluidics which provided for greatly reduced flow rates [124, 125]. Fluid flow through such early devices tended to be passive, established through the movement of fluid from large to small reservoirs, or active through peristaltic pumps (though volume control was limited in the latter approach). In order to facilitate greater control over fluid movement, Takayama and Smith established a computer-controlled, integrated fluid-control system utilizing up to hundreds of on-chip pumps and valves, driven by individually actuated Braille pins, which effectively squeezed fluid through individual channels. Using such an approach to establish a dynamic flow environment around the embryo (facilitated by the movement of media, but without the exchange of media) it was observed that mouse blastocyst cell number and subsequent implantation rate were significantly increased [126]. However, it is potentially through novel microfabrication approaches that such perfusion systems will be able to be evaluated in a clinical setting.

Microfabrication

As previously described, microfabrication of microfluidic devices for ART has been typically moulded from polydimethylsiloxane (PDMS) [38]. This material has been preferred due to its transparency, biocompatibility, and gas permeability. Nevertheless, the drive for sub-micron fluidic structures that have precise geometries and scalability for manufacturing is constantly being pursued; alternatives to PDMS are being sought. A major limitation from a cell and embryo culture perspective is the absorption and evaporation of media within microchannels of PDMS microfluidic devices [127, 128]. Furthermore, PDMS-moulded features do not have the resolution to create interchanging parts. An alternative is micron 3D-printable glass, now provided commercially from

sources such as Glassomer GmbH (Germany). A technology also capable of sub-micron feature 3D printing is two-photon polymerization (2PP). 2PP printing is a high-resolution micro additive manufacturing technique using photosensitive polymers. Similar in principle to 2-photon fluorescence microscopy, two-photon absorption (2PA) creates a nonlinear energy distribution centred at the laser focal point of two long-wavelength laser sources [129]. At that point, 2PA excitation induces UV-sensitive monomer crosslinking of the polymer. Consequently, 2PP can fabricate precise structures with high-resolution features, smaller than the wavelength of the laser, thereby creating devices with feature sizes in the sub-micron range [130]. Further, 2PP supports the use of photopolymers that are biocompatible and non-cytotoxic for cell culture applications following appropriate post-printing treatment, which has been demonstrated with a comparable printing technique called digital light processing [131].

Two recent publications reveal how versatile 2PP fabrication is for creating devices for use within IVF laboratories. The first describes a device for intracytoplasmic sperm injection (ICSI) [132]. The design of this device removed the need for a holding pipette, thereby removing at least one micromanipulator and pressure controller from an ICSI workstation. The oocyte is held within a cavity of a two-piece device created by 2PP, with dimensions and geometries unachievable with other manufacturing techniques. The linear array of chambers provides traceability of oocytes before and after injection, reducing the risk of failure to inject or double injection under conventional ICSI systems. The second publication [133] reports that 2PP devices are entirely suitable as cryopreservation devices. Similar cryo-survival and subsequent development rates were obtained following vitrification of mouse oocytes and embryos. An advantage of 2PP for this application is the minimal exposure volumes of cryo-protectants that such structures can impart.

Application of 2PP in microfluidics has been hampered by limitations in the time taken for printing and the size of print achievable. This is being rapidly resolved with further development of commercial 3D 2PP printers from companies such as Nanoscribe GmbH (Germany) and UpNano (Austria). For example, the NanoOne (UpNano) has a horizontal print capacity of 1 cm². This can support several microfluidic channels and has now been developed for microfluidic culture of oocytes and embryos [134]. This publication demonstrated how 2PP can print two disparate fluidic parts (“nest” and “cradle”; **Figure 28.6**) that interlock through printed features such as a nozzle barb connector. The device supports oocyte–cumulus expansion and embryo development (**Figure 28.7**) under dynamic flow. As such, a new era of microfluidic devices centred on the high resolution and manufacturing capacity of 2PP micro 3D printing in various polymers and glass is rapidly emerging (**Figures 28.6** and **28.7**).

Not only does this breakthrough in microfabrication offer exciting new possibilities for performing *in vitro* maturation and embryo culture but also could lead to the development of an automated means of performing vitrification, as initially proposed by [135], whereby the cryopreservation solutions are introduced in a gradient fashion made feasible through built-in valving in the microfluidic device itself.

Summary

Microfluidics has held great promise for assisted human conception for more than 20 years. However, recent developments in this field, including novel microfabrication approaches, indicate that

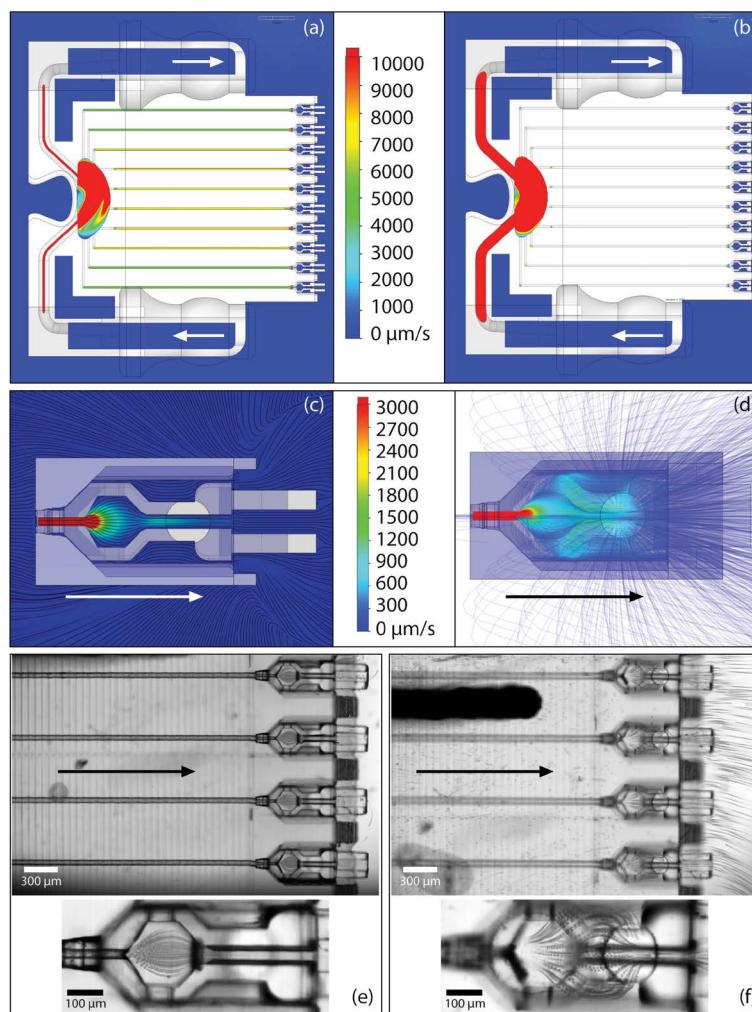


FIGURE 28.6 Predicted and actual flow patterns through the “nest”–“cradle” interlocking 2PP printed device: (a) computational fluid dynamic (CFD) modelling showing distribution of flow across the nest channels; (b) CFD modelling at nest surface showing flow through the inlet, back reservoir, and outlet located 250 µm above the centre of the channels; (c) CFD modelling showing the smoothing effect of the nozzle within an individual cradle; (d) CFD modelling of the flow trajectories within an individual cradle from below; (e) projection of bead tracks from above with the focal plane set at the centre of the channels; (f) projection of bead tracks from above with the focal plane set to nest top surface. (Scale bars (a)–(b) = 1 mm; (c)–(d) cradle length from the nozzle to opposite end = 1 mm; (e)–(f) = as labelled; arrows indicate the direction of fluid flow.)

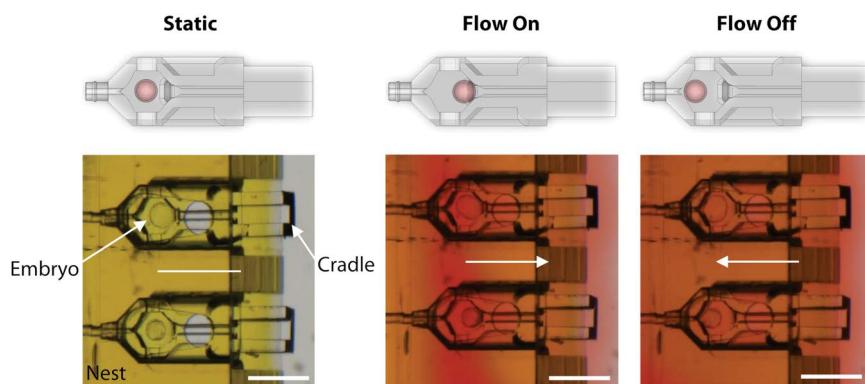


FIGURE 28.7 Mouse embryo movement in a changing dynamic flow environment with the introduction of red stained medium: (a) static conditions show the expanded blastocysts housed inside the cradles and nest after 48 hours of culture; (b) Flow On shows the embryos move to the right as the flow pushed them to the back of the cell chamber; (c) Flow Off shows the embryos move to the left back towards the nozzle. (Scale bars = 300 µm.)

we are finally on the verge of seeing these technologies being clinically validated for a wide range of tasks in infertility treatment, from sperm preparation and diagnosis, ICSI, embryo culture, and finally vitrification. Furthermore, all of these approaches lend themselves towards automation (in total or in part) of several key procedures in the IVF laboratory [136], thereby greatly reducing operator variability inherent in these technically demanding tasks. This in turn will lead to improved efficiencies and efficacies in the IVF laboratory and improve standardization in lab processes, culminating in reduced time to pregnancy and increased pregnancy rates. Ultimately, this will increase the accessibility of IVF to more patients worldwide [137].

References

- Kashaninejad N, Shiddiky MJA, Nguyen NT. Advances in microfluidics-based assisted reproductive technology: From sperm sorter to reproductive system-on-a-chip. *Adv Biosyst.* 2018;2(3):1700197.
- Zhang J, Yan S, Yuan D, Alici G, Nguyen N-T, Warkiani ME, et al. Fundamentals and applications of inertial microfluidics: A review. *Lab Chip.* 2016; 16(1): 10–34.
- Bai Y, Gao M, Wen L, He C, Chen Y, Liu C, et al. Applications of microfluidics in quantitative biology. *Biotechnol J.* 2018; 13(5): 1700170.
- Bohr A, Colombo S, Jensen H. Future of microfluidics in research and in the market. *Microfluidics for Pharmaceutical Applications.* Amsterdam, Netherlands: Elsevier, pp. 425–65, 2019.
- Chaudhuri PK, Warkiani ME, Jing T, Lim CT. Microfluidics for research and applications in oncology. *Analyst.* 2016;141(2):504–24.
- Chen J, Chen D, Xie Y, Yuan T, Chen X. Progress of microfluidics for biology and medicine. *Nano-Micro Lett.* 2013;5(1):66–80.
- Niculescu A-G, Chircov C, Bîrcă AC, Grumezescu AM. Fabrication and applications of microfluidic devices: A review. *Int J Mol Sci.* 2021;22(4):2011.
- Razavi Bazaz S, Rouhi O, Raoufi MA, Ejeian F, Asadnia M, Jin D, et al. 3D printing of inertial microfluidic devices. *Sci Rep.* 2020;10(1):1–14.
- Scott SM, Ali Z. Fabrication methods for microfluidic devices: An overview. *Micromachines.* 2021;12(3):319.
- Vasilescu SA, Bazaz SR, Jin D, Shimon O, Warkiani ME. 3D printing enables the rapid prototyping of modular microfluidic devices for particle conjugation. *Appl Mater Today.* 2020;20:100726.
- Sharma B, Sharma A. Microfluidics: Recent advances toward lab-on-chip applications in bioanalysis. *Adv Eng Mater.* 2022; 24(2):2100738.
- Terry SC, Jerman JH, Angell JB. A gas chromatographic air analyzer fabricated on a silicon wafer. *IEEE Trans Electron Devices.* 1979;26(12):1880–6.
- Nosrati R, Gong MM, San Gabriel MC, Pedraza CE, Zini A, Sinton D. Paper-based quantification of male fertility potential. *Clin Chem.* 2016;62(3):458–65.
- Ortseifen V, Viehues M, Wobbe L, Grünberger A. Microfluidics for biotechnology: Bridging gaps to foster microfluidic applications. *Front Bioeng Biotechnol.* 2020;8:589074.
- Panesar S, Neethirajan S. Microfluidics: Rapid diagnosis for breast cancer. *Nano-Micro Lett.* 2016;8(3):204–20.
- Sun W, Luo Z, Lee J, Kim HJ, Lee K, Tebon P, et al. Organ-on-a-chip for cancer and immune organs modeling. *Adv Healthcare Mat.* 2019;8(4):1801363.
- Eamer L, Vollmer M, Nosrati R, San Gabriel MC, Zeidan K, Zini A, et al. Turning the corner in fertility: High DNA integrity of boundary-following sperm. *Lab Chip.* 2016;16(13):2418–22.
- Nosrati R, Graham PJ, Zhang B, Riordon J, Lagunov A, Hannam TG, et al. Microfluidics for sperm analysis and selection. *Nat Rev Urol.* 2017;14(12):707–30.
- Zargari S, Veladi H, Sadeghzadeh B, Shahabi P, Frounchi J. A microfluidic chip for in vitro oocyte maturation. *Sensor Lett.* 2016;14(4):435–40.
- Zhao G, Zhang Z, Zhang Y, Chen Z, Niu D, Cao Y, et al. A microfluidic perfusion approach for on-chip characterization of the transport properties of human oocytes. *Lab Chip.* 2017;17(7):1297–305.
- Nosrati R, Vollmer M, Eamer L, San Gabriel MC, Zeidan K, Zini A, et al. Rapid selection of sperm with high DNA integrity. *Lab Chip.* 2014;14(6):1142–50.
- Vasilescu SA, Khorsandi S, Ding L, Bazaz SR, Nosrati R, Gook D, et al. A microfluidic approach to rapid sperm recovery from heterogeneous cell suspensions. *Sci Rep.* 2021;11(1):7917.
- Zhao G, Fu J. Microfluidics for cryopreservation. *Biotechnol Adv.* 2017;35(2):323–36.
- Ko Y-J, Maeng J-H, Hwang SY, Ahn Y. Design, fabrication, and testing of a microfluidic device for thermotaxis and chemotaxis assays of sperm. *SLAS Technol.* 2018;23(6):507–15.
- Nishat S, Jafry AT, Martinez AW, Awan FR. Paper-based based microfluidics: Simplified fabrication and assay methods. *Sens Actuators B. Chem.* 2021;336:129681.
- Gargus ES, Rogers HB, McKinnon KE, Edmonds ME, Woodruff TK. Engineered reproductive tissues. *Nat Biomed Eng.* 2020; 4(4):381–93.
- Mancini V, Pensabene V. Organs-on-chip models of the female reproductive system. *Bioengineering.* 2019;6(4):103.
- Xiao S, Coppeta JR, Rogers HB, Isenberg BC, Zhu J, Olalekan SA, et al. A microfluidic culture model of the human reproductive tract and 28-day menstrual cycle. *Nat Commun.* 2017;8(1):1–13.
- Lee SH, Sung JH. Organ-on-a-chip technology for reproducing multiorgan physiology. *Adv Healthcare Mater.* 2018;7(2):1700419.
- Manz A, Gruber N, Widmer H. Miniaturized total chemical analysis systems: A novel concept for chemical sensing. *Sens Actuators B. Chem.* 1990;1(1-6):244–8.
- Mohammed MI, Haswell S, Gibson I. Lab-on-a-chip or chip-in-a-lab: Challenges of commercialization lost in translation. *Procedia Technol.* 2015;20:54–9.
- Inhorn MC. Masturbation, semen collection and men's IVF experiences: Anxieties in the Muslim world. *Body Soc.* 2007;13(3): 37–53.
- Alvarez C, Castilla J, Martinez L, Ramírez J, Vergara F, Gaforio J. Biological variation of seminal parameters in healthy subjects. *Hum Reprod.* 2003;18(10):2082–8.
- Liu Y, Chesnut M, Guitreau A, Beckham J, Melvin A, Eades J, et al. Microfabrication of low-cost customisable counting chambers for standardised estimation of sperm concentration. *Reprod Fertil Dev.* 2020;32(9):873.
- Kime D, Ebrahimi M, Nysten K, Roelants I, Rurangwa E, Moore H, et al. Use of computer assisted sperm analysis (CASA) for monitoring the effects of pollution on sperm quality of fish: Application to the effects of heavy metals. *Aquatic Toxicol.* 1996;36(3–4): 223–37.
- WHO. *Laboratory Manual for the Examination and Processing of Human Semen.* 6th edition. Geneva, Switzerland: World Health Organisation, p. 276, 2021.
- Pérez-Cerezales S, Miranda A, Gutiérrez-Adán A. Comparison of four methods to evaluate sperm DNA integrity between mouse caput and cauda epididymidis. *Asian J Androl.* 2012;14(2):335–7.
- Whitesides GM. The origins and the future of microfluidics. *Nature.* 2006;442(7101):368–73.
- Chen C-Y, Chiang T-C, Lin C-M, Lin S-S, Jong D-S, Tsai VF-S, et al. Sperm quality assessment via separation and sedimentation in a microfluidic device. *Analyst.* 2013;138(17):4967–74.
- McCormack MC, McCallum S, Behr B. A novel microfluidic device for male subfertility screening. *J Urol.* 2006;175(6):2223–7.
- Coppola M, Klotz K, Kim K-a, Cho H, Kang J, Shetty J, et al. SpermCheck® fertility, an immunodiagnostic home test that detects normozoospermia and severe oligozoospermia. *Hum Reprod.* 2010;25(4):853–61.

42. Björndahl L, Kirkman-Brown J, Hart G, Rattle S, Barratt CLR. Development of a novel home sperm test. *Hum Reprod.* 2006; 21(1):145–9.
43. Schaff UY, Fredriksen LL, Epperson JG, Quebral TR, Naab S, Sarno MJ, et al. Novel centrifugal technology for measuring sperm concentration in the home. *Fertil Steril.* 2017;107(2):358–64.e4.
44. Gong MM, Nosrati R, San Gabriel MC, Zini A, Sinton D. Direct DNA analysis with paper-based ion concentration polarization. *J Am Chem Soc.* 2015;137(43):13913–9.
45. de Wagenaar B, Dekker S, de Boer HL, Bomer JG, Olthuis W, van den Berg A, et al. Towards microfluidic sperm refinement: Impedance-based analysis and sorting of sperm cells. *Lab Chip.* 2016;16(8):1514–22.
46. Segerink LI, Sprinkels AJ, ter Braak PM, Vermeij I, Van den Berg A. On-chip determination of spermatozoa concentration using electrical impedance measurements. *Lab Chip.* 2010;10(8):1018–24.
47. Sun Y, Haglund TA, Rogers AJ, Ghanim AF, Sethu P. Microfluidics technologies for blood-based cancer liquid biopsies. *Analytica Chimica Acta.* 2018;1012:10–29.
48. Contreras-Naranjo JC, Wu HJ, Ugaz VM. Microfluidics for exosome isolation and analysis: Enabling liquid biopsy for personalized medicine. *Lab Chip.* 2017;17(21):3558–77.
49. Murdica V, Giacomini E, Alteri A, Bartolacci A, Cermisoni GC, Zarovni N, et al. Seminal plasma of men with severe asthenozoospermia contain exosomes that affect spermatozoa motility and capacitation. *Fertil Steril.* 2019;111(5):897–908.e2.
50. Murdica V, Cermisoni GC, Zarovni N, Salonia A, Vigano P, Vago R. Proteomic analysis reveals the negative modulator of sperm function glycoprotein as over-represented in semen exosomes isolated from asthenozoospermic patients. *Hum Reprod.* 2019;34(8):1416–27.
51. Barceló M, Castells M, Bassas L, Vigués F, Larriba S. Semen miRNAs contained in exosomes as non-invasive biomarkers for prostate cancer diagnosis. *Sci Rep.* 2019;9(1):1–16.
52. Barceló M, Mata A, Bassas L, Larriba S. Exosomal microRNAs in seminal plasma are markers of the origin of azoospermia and can predict the presence of sperm in testicular tissue. *Hum Reprod.* 2018;33(6):1087–98.
53. Goss DM, Vasilescu SA, Sacks G, Gardner DK, Warkiani ME. Microfluidics facilitating the use of small extracellular vesicles in innovative approaches to male infertility. *Nat Rev Urol.* 2022;20(2):66–95.
54. Harding CV, Heuser JE, Stahl PD. Exosomes: Looking back three decades and into the future. *J Cell Biol.* 2013;200(4):367–71.
55. Zheng R, Du M, Wang X, Xu W, Liang J, Wang W, et al. Exosome-transmitted long non-coding RNA PTENP1 suppresses bladder cancer progression. *Mol Cancer.* 2018;17(1):1–13.
56. Pegtel DM, Gould SJ. Exosomes. *Annu Rev Biochem.* 2019; 88:487–514.
57. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol.* 2007;9(6):654–9.
58. Men Y, Yelick J, Jin S, Tian Y, Chiang MSR, Higashimori H, et al. Exosome reporter mice reveal the involvement of exosomes in mediating neuron to astroglia communication in the CNS. *Nat Commun.* 2019;10(1):1–18.
59. Yang C, Guo WB, Zhang WS, Bian J, Yang JK, Zhou QZ, et al. Comprehensive proteomics analysis of exosomes derived from human seminal plasma. *Andrology.* 2017;5(5):1007–15.
60. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol.* 2006;30(1):3.22. 1-3. 9.
61. Bruus H. Acoustofluidics 7: The acoustic radiation force on small particles. *Lab Chip.* 2012;12(6):1014–21.
62. Yang D, Zhang W, Zhang H, Zhang F, Chen L, Ma L, et al. Progress, opportunity, and perspective on exosome isolation-efforts for efficient exosome-based theranostics. *Theranostics* 2020;10(8):3684–7.
63. Suwatthanarak T, Thiodorus IA, Tanaka M, Shimada T, Takeshita D, Yasui T, et al. Microfluidic-based capture and release of cancer-derived exosomes via peptide–nanowire hybrid interface. *Lab Chip.* 2021;21(3):597–607.
64. Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H, et al. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod.* 2003;18(5): 1023–8.
65. Coughlan C, Clarke H, Cutting R, Saxton J, Waite S, Ledger W, et al. Sperm DNA fragmentation, recurrent implantation failure and recurrent miscarriage. *Asian J Androl.* 2015;17(4):681.
66. Henkel R, Kierspel E, Hajimohammad M, Stalf T, Hoogendoijk C, Mehnert C, et al. DNA fragmentation of spermatozoa and assisted reproduction technology. *Reprod Biomed Online.* 2003; 7(4):477–84.
67. Sedó CA, Bilinski M, Lorenzi D, Uriondo H, Noblia F, Longobucco V, et al. Effect of sperm DNA fragmentation on embryo development: Clinical and biological aspects. *JBRA Assist Reprod.* 2017;21(4):343.
68. De Martin H, Miranda EP, Cocuzza MS, Monteleone PA. Density gradient centrifugation and swim-up for ICSI: Useful, unsafe, or just unsuitable? *J Assist Reprod Genet.* 2019;36(12):2421–3.
69. Malvezzi H, Sharma R, Agarwal A, Abuzeinadah AM, Abu-Elmagd M. Sperm quality after density gradient centrifugation with three commercially available media: A controlled trial. *Reprod Biol Endocrinol.* 2014;12(1):1–7.
70. Aitken R, Bronson R, Smith T, De Iuliis G. The source and significance of DNA damage in human spermatozoa; A commentary on diagnostic strategies and straw man fallacies. *Mol Hum Reprod.* 2013;19(8):475–85.
71. Rappa KL, Rodriguez HF, Hakkarainen GC, Anchan RM, Mutter GL, Asghar W. Sperm processing for advanced reproductive technologies: Where are we today? *Biotechnol Adv.* 2016;34(5): 578–87.
72. Leung ET, Lee C-L, Tian X, Lam KK, Li RH, Ng EH, et al. Simulating nature in sperm selection for assisted reproduction. *Nat Rev Urol.* 2022;19(1):16–36.
73. Sakkas D, Ramalingam M, Garrido N, Barratt CL. Sperm selection in natural conception: What can we learn from mother nature to improve assisted reproduction outcomes? *Hum Reprod Update.* 2015;21(6):711–26.
74. Wang C, Swerdlow RS. Limitations of semen analysis as a test of male fertility and anticipated needs from newer tests. *Fertil Steril.* 2014;102(6):1502–7.
75. Asghar W, Velasco V, Kingsley JL, Shoukat MS, Shafiee H, Anchan RM, et al. Selection of functional human sperm with higher DNA integrity and fewer reactive oxygen species. *Adv Healthcare Mater.* 2014;3(10):1671–9.
76. Cho BS, Schuster TG, Zhu X, Chang D, Smith GD, Takayama S. Passively driven integrated microfluidic system for separation of motile sperm. *Anal Chem.* 2003;75(7):1671–5.
77. Lopez-Garcia MC, Monson R, Haubert K, Wheeler M, Beebe D. Sperm motion in a microfluidic fertilization device. *Biomed Microdevices.* 2008;10(5):709–18.
78. Tasoglu S, Safaei H, Zhang X, Kingsley JL, Catalano PN, Gurkan UA, et al. Exhaustion of racing sperm in nature-mimicking microfluidic channels during sorting. *Small.* 2013;9(20):3374–84.
79. Schuster TG, Cho B, Keller LM, Takayama S, Smith GD. Isolation of motile spermatozoa from semen samples using microfluidics. *Reprod Biomed Online.* 2003;7(1):75–81.
80. Shiota K, Yotsumoto F, Itoh H, Obama H, Hidaka N, Nakajima K, et al. Separation efficiency of a microfluidic sperm sorter to minimize sperm DNA damage. *Fertil Steril.* 2016;105(2):315–21.e1.
81. Yetkinel S, Kilicdag EB, Aytac PC, Haydardegedoglu B, Simsek E, Cok T. Effects of the microfluidic chip technique in sperm selection for intracytoplasmic sperm injection for unexplained infertility: A prospective, randomized controlled trial. *J Assist Reprod Genet.* 2019;36(3):403–9.

82. Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL. Evidence for the function of hyperactivated motility in Sperm. *Biol Reprod.* 1991;44(2):375–81.
83. Yazdan Parast F, O'Bryan MK, Nosrati R. Sperm syringe: 3D sorting platform for assisted reproduction. *Adv Mater Technol.* 2022;44(2):2101291.
84. Ma R, Xie L, Han C, Su K, Qiu T, Wang L, et al. In vitro fertilization on a single-oocyte positioning system integrated with motile sperm selection and early embryo development. *Anal Chem.* 2011; 83(8): 2964–70.
85. Ko Y-J, Maeng J-H, Lee B-C, Lee S, Hwang SY, Ahn Y. Separation of progressive motile sperm from mouse semen using on-chip chemotaxis. *Anal Sci.* 2012;28(1):27.
86. Ainsworth C, Nixon B, Aitken RJ. Development of a novel electrophoretic system for the isolation of human spermatozoa. *Hum Reprod.* 2005;20(8):2261–70.
87. Li Z, Liu W, Qiu T, Xie L, Chen W, Liu R, et al. The construction of an interfacial valve-based microfluidic chip for thermotaxis evaluation of human sperm. *Biomicrofluidics.* 2014;8(2):024102.
88. Pérez-Cerezales S, Laguna-Barraza R, de Castro AC, Sánchez-Calabuig MJ, Cano-Oliva E, de Castro-Pita FJ, et al. Sperm selection by thermotaxis improves ICSI outcome in mice. *Sci Rep.* 2018;8(1):1–14.
89. Suarez SS. Mammalian sperm interactions with the female reproductive tract. *Cell Tissue Res.* 2016;363(1):185–94.
90. Simon L, Murphy K, Aston KI, Emery BR, Hotaling JM, Carrell DT. Optimization of microelectrophoresis to select highly negatively charged sperm. *J Assist Reprod Genet.* 2016;33(6): 679–88.
91. Fleming S, Ilad R, Griffin AG, Wu Y, Ong K, Smith H, et al. Prospective controlled trial of an electrophoretic method of sperm preparation for assisted reproduction: Comparison with density gradient centrifugation. *Hum Reprod.* 2008;23(12):2646–51.
92. Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: A systematic review. *Hum Reprod Update.* 2011;17(6):719–33.
93. Flannigan R, Bach PV, Schlegel PN. Microdissection testicular sperm extraction. *Transl Androl Urol.* 2017;6(4):745–52.
94. Esteves SC. Clinical management of infertile men with nonobstructive azoospermia. *Asian J Androl.* 2015;17(3):459–70.
95. Samuel R, Badamjav O, Murphy KE, Patel DP, Son J, Gale BK, et al. Microfluidics: The future of microdissection TESE? *Syst Biol Reprod Med.* 2016;62(3):161–70.
96. Samuel R, Son J, Jenkins TG, Jafek A, Feng H, Gale BK, et al. Microfluidic system for rapid isolation of sperm from microdissection TESE specimens. *Urology.* 2020;140:70–6.
97. Son J, Jafek AR, Carrell DT, Hotaling JM, Gale BK. Sperm-like-particle (SLP) behavior in curved microfluidic channels. *Microfluid Nanofluid.* 2019;23(1):4.
98. Son J, Murphy K, Samuel R, Gale BK, Carrell DT, Hotaling JM. Non-motile sperm cell separation using a spiral channel. *Anal Methods.* 2015;7(19):8041–7.
99. Son J, Samuel R, Gale BK, Carrell DT, Hotaling JM. Separation of sperm cells from samples containing high concentrations of white blood cells using a spiral channel. *Biomicrofluidics.* 2017; 11(5):054106.
100. Anbari F, Khalili MA, Sultan Ahamed AM, Mangoli E, Nabi A, Dehghanpour F, et al. Microfluidic sperm selection yields higher sperm quality compared to conventional method in ICSI program: A pilot study. *Syst Biol Reprod Med.* 2021;67(2):137–43.
101. Gode F, Bodur T, Gunturkun F, Gurbuz AS, Tamer B, Pala I, et al. Comparison of microfluidic sperm sorting chip and density gradient methods for use in intrauterine insemination cycles. *Fertil Steril.* 2019;112(5):842–8.e1.
102. Parrella A, Keating D, Cheung S, Xie P, Stewart JD, Rosenwaks Z, et al. A treatment approach for couples with disrupted sperm DNA integrity and recurrent ART failure. *J Assist Reprod Genet.* 2019;36(10):2057–66.
103. Zheng W, Jiang X. Synthesizing living tissues with microfluidics. *Acc Chem Res.* 2018;51(12):3166–73.
104. Shrestha J, Razavi Bazaz S, Aboulkheyr Es H, Yaghobian Azari D, Thierry B, Ebrahimi Warkiani M, et al. Lung-on-a-chip: The future of respiratory disease models and pharmacological studies. *Crit Rev Biotechnol.* 2020;40(2):213–30.
105. Baert Y, Ruetschle I, Cools W, Oehme A, Lorenz A, Marx U, et al. A multi-organ-chip co-culture of liver and testis equivalents: A first step toward a systemic male reprotoxicity model. *Hum Reprod.* 2020;35(5):1029–44.
106. Sharma S, Venzac B, Burgers T, Schlatt S, Le Gac S. Testis-on-chip platform to study ex vivo primate spermatogenesis and endocrine dynamics. *Organs-on-a-Chip.* 2022;4:100023.
107. Komeya M, Kimura H, Nakamura H, Yokonishi T, Sato T, Kojima K, et al. Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. *Sci Rep.* 2016; 6(1):1–10.
108. Yamanaka H, Komeya M, Nakamura H, Sanjo H, Sato T, Yao M, et al. A monolayer microfluidic device supporting mouse spermatogenesis with improved visibility. *Biochem Biophys Res Commun.* 2018;500(4):885–91.
109. Komeya M, Hayashi K, Nakamura H, Yamanaka H, Sanjo H, Kojima K, et al. Pumpless microfluidic system driven by hydrostatic pressure induces and maintains mouse spermatogenesis in vitro. *Sci Rep.* 2017;7(1):1–8.
110. Ferraz MA, Henning HH, Costa PF, Malda J, Melchels FP, Wubbolt R, et al. Improved bovine embryo production in an oviduct-on-a-chip system: Prevention of poly-spermic fertilization and parthenogenic activation. *Lab Chip.* 2017; 17(5): 905–16.
111. Ulbrich SE, Zitta K, Hiendleder S, Wolf E. In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. *Theriogenology.* 2010;73(6):802–16.
112. Gualtieri R, Mollo V, Braun S, Barbato V, Fiorentino I, Talevi R. Bovine oviductal monolayers cultured under three-dimension conditions secrete factors able to release spermatozoa adhering to the tubal reservoir in vitro. *Theriogenology.* 2013; 79(3):429–35.
113. Gnecco JS, Pensabene V, Li DJ, Ding T, Hui EE, Bruner-Tran KL, et al. Compartmentalized culture of perivascular stroma and endothelial cells in a microfluidic model of the human endometrium. *Ann Biomed Eng.* 2017;45(7):1758–69.
114. Wei-Xuan L, Liang G-T, Wei Y, Zhang Q, Wei W, Xiao-Mian Z, et al. Artificial uterus on a microfluidic chip. *Chinese J Anal Chem.* 2013;41(4):467–72.
115. Mizuno J, Ostrovodov S, Sakai Y, Fujii T, Nakamura H, Inui H. Human ART on chip: Improved human blastocyst development and quality with IVF-chip. *Fertil Steril.* 2007;88:S101.
116. Chang K-W, Chang P-Y, Huang H-Y, Li C-J, Tien C-H, Yao D-J, et al. Womb-on-a-chip biomimetic system for improved embryo culture and development. *Sens Actuators B. Chem.* 2016;226:218–26.
117. Park S-R, Kim S-R, Lee JW, Park CH, Yu W-J, Lee S-J, et al. Development of a novel dual reproductive organ on a chip: Recapitulating bidirectional endocrine crosstalk between the uterine endometrium and the ovary. *Biofabrication.* 2020;13(1): 015001.
118. Bhise NS, Ribas J, Manoharan V, Zhang YS, Polini A, Massa S, et al. Organ-on-a-chip platforms for studying drug delivery systems. *J Control Release.* 2014;190:82–93.
119. Zheng F, Fu F, Cheng Y, Wang C, Zhao Y, Gu Z. Organ-on-a-Chip systems: Microengineering to biomimic living systems. *Small.* 2016;12(17):2253–82.
120. Zhang B, Korolj A, Lai BFL, Radisic M. Advances in organ-on-a-chip engineering. *Nat Rev Mater.* 2018;3(8):257–78.
121. Gardner DK. Human embryo development and assessment of viability. In: Encyclopedia of Reproduction, 2. 2nd edition, Skinner M, (ed.). Elsevier, pp. 176–5, 2018.
122. Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int.* 1994;18(12):1163–80.

123. Lim J, Reggio B, Godke R, Hansel W. Perfusion culture system for bovine embryos: Improvement of embryo development by use of bovine oviduct epithelial cells, an antioxidant and polyvinyl alcohol. *Reprod Fertil Dev.* 1997;9(4):411–8.
124. Glasgow IK, Zeringue HC, Beebe DJ, Choi S-J, Lyman JT, Chan NG, et al. Handling individual mammalian embryos using microfluidics. *IEEE Trans Biomed Eng.* 2001;48(5):570–8.
125. Beebe D, Wheeler M, Zeringue H, Walters E, Raty S. Microfluidic technology for assisted reproduction. *Theriogenology.* 2002;57(1): 125–35.
126. Heo Y, Cabrera L, Bormann C, Shah C, Takayama S, Smith G. Dynamic microfunnel culture enhances mouse embryo development and pregnancy rates. *Hum Reprod.* 2010;25(3):613–22.
127. Heo YS, Cabrera LM, Song JW, Futai N, Tung Y-C, Smith GD, et al. Characterization and resolution of evaporation-mediated osmolality shifts that constrain microfluidic cell culture in poly(dimethylsiloxane) devices. *Anal Chem.* 2007;79(3):1126–34.
128. Halldorsson S, Lucumi E, Gómez-Sjöberg R, Fleming RM. Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. *Biosens Bioelectron.* 2015;63:218–31.
129. Faraji Rad Z, Prewett PD, Davies GJ. High-resolution two-photon polymerization: The most versatile technique for the fabrication of microneedle arrays. *Microsyst Nanoeng.* 2021;7(1):1–17.
130. Hu Q, Rance GA, Trindade GF, Pervan D, Jiang L, Foerster A, et al. The influence of printing parameters on multi-material two-photon polymerisation based micro additive manufacturing. *Addit Manuf.* 2022;51:102575.
131. González G, Baruffaldi D, Martinengo C, Angelini A, Chiappone A, Roppolo I, et al. Materials testing for the development of biocompatible devices through vat-polymerization 3D printing. *Nanomaterials.* 2020;10(9):1788.
132. Yagoub SH, Thompson JG, Orth A, Dholakia K, Gibson BC, Dunning KR. Fabrication on the microscale: A two-photon polymerized device for oocyte microinjection. *J Assist Reprod Genet.* 2022;39(7):1503–13.
133. Yagoub SH, Lim M, Tan TC, Chow DJ, Dholakia K, Gibson BC, et al. Vitrification within a nanoliter volume: Oocyte and embryo cryopreservation within a 3D photopolymerized device. *J Assist Reprod Genet.* 2022;39(9):1997–2014.
134. McLennan HJ, Blanch AJ, Wallace SJ, Ritter LJ, Heinrich SL, Gardner DK, et al. Nano-liter perfusion microfluidic device made entirely by two-photon polymerization for dynamic cell culture with easy cell recovery. *Sci Rep.* 2023;13(1):1–16.
135. Heo YS, Lee H-J, Hassell BA, Irimia D, Toth TL, Elmoazzen H, et al. Controlled loading of cryoprotectants (CPAs) to oocyte with linear and complex CPA profiles on a microfluidic platform. *Lab Chip* 2011;11(20):3530–7.
136. Gardner DK. The way to improve ART outcomes is to introduce more technologies in the laboratory. *Reprod BioMed Online.* 2022;44(3):389–92.
137. Gardner DK, Sakkas D. Making and selecting the best embryo in the laboratory. *Fertil Steril.* 2022. <https://doi.org/10.1016/j.fertnstert.2022.11.007>.

GENOME EDITING IN HUMAN REPRODUCTION

Helen C. O'Neill

Clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing is a revolutionary technology that allows for precise and efficient manipulation of DNA sequences. It has rapidly become one of the most powerful tools in molecular biology, with a wide range of applications in areas such as agriculture, medicine, and biotechnology and with expanding editing capabilities. One of the most promising areas of research for CRISPR is human health.

The CRISPR-Cas system is a prokaryotic adaptive immune system that utilizes small RNAs, called guide RNAs (gRNAs), to target and cleave specific DNA sequences. The gRNA guides a nuclease, usually Cas9, to a specific location on the genome, where it can make a double-stranded break (DSB) in the DNA. The DSB can then be repaired by the cell's own repair machinery, leading to changes in the DNA sequence. These changes can include insertions, deletions, or substitutions of nucleotides, which can be used to correct genetic mutations or disrupt the function of specific genes. CRISPR knockout, CRISPR activation, and CRISPR interference-based genetic screens also offer the opportunity to assess functions of thousands of genes in massively parallel assays [1].

Human genome editing holds tremendous potential for the treatment and prevention of disease. Beyond clinical applications, human germline genome editing would permit unprecedented investigation into gene function and cell fate in human embryogenesis; allowing resolution of the elusive mechanisms that underpin pre-implantation human development [2]. Genome editing systems permit targeted gene disruption or modification in the living cells of almost all organisms [3], the molecular tools for human genome editing are now readily available [4, 5]. These technologies are amenable to both somatic and germline cells. CRISPR/Cas editing systems have already been successfully trialled in human somatic cell editing, including improving anti-tumour immunity in cancer patient T cells [6, 7]. However, human germline genome editing has only been conducted in a handful of experiments [8]. The insights from many of these studies have been limited, as many original studies used non-viable triploid (3PN) embryos to circumvent ethical challenges.

The advancement of human germline genome editing remains unequivocally controversial and evokes several long-standing, significant social and bioethical objections, including regulatory considerations regarding the intent of use, specifically in the use of genome editing for enhancement; concerns surrounding ethnic representation in samples; and ensuring equity of access [9]. Yet, debates surrounding the ethical ramifications of human germline editing are reduced if safe and efficacious protocols for practice can be established. Currently, germline genome editing experiments may risk the introduction of potentially dangerous, heritable changes to the human genome, but also have the potential to correct devastating familial mutations. Most regard

previous attempts at human genome editing as premature and irresponsible [10]. Off-target editing, unintentional chromosomal rearrangements, and mosaicism persist as adverse, but not infrequent, outcomes of CRISPR/Cas9 editing experiments [11, 12], but these are overcome with newer methods of genome editing. The timing and delivery methods through which editing components are introduced to target cells are key determinants of experimental outcomes. However, there is a paucity of information regarding best practice for introduction of CRISPR/Cas systems to large animal and human cells and embryos and therefore little information about what different editing methods could mean in terms of success.

CRISPR/Cas genome editing mechanisms

CRISPR/Cas editing experiments rely on two components: a single guide RNA (sgRNA) and a Cas endonuclease [13, 14]. The sgRNA consists of a "scaffold" trans-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) (Figure 29.1a) [3]. The "spacer" region of crRNA shares 17–20 nt homology with the target sequence, which is located a proximal to the protospacer adjacent motif (PAM) [15]. The PAM, which can be as short as a trinucleotide sequence, functions as the Cas binding site and signals for target site-specific DNA cleavage (Figure 29.1b) [16]. The resulting double-strand break (DSB) can be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Figure 29.1c) [17, 18]. Predominantly used for gene knockouts, NHEJ utilizes native, error-prone DNA repair machinery to resolve the DSB; here, small insertions or deletions (indels) result in gene disruption [17, 19]. Alternatively, a single-stranded oligodeoxynucleotide (ssODN) template with homology to the target region can be delivered with the Cas enzyme to initiate HDR and introduce specific changes to the target sequence, including gene knock-ins or point mutations. However, HDR typically occurs at a lower propensity than NHEJ-mediated repair [20].

Streptococcus pyogenes Cas9 ("Cas9") remains the most widely used Cas enzyme [3, 21]. However, the early success of Cas9 propelled efforts to diversify the potential applications of CRISPR-based editing systems. Subsequently, several novel endonuclease-directed systems for gene targeting have been identified, including Cas12a, Cas13a, and LbCpf1 [22]. Each endonuclease recognizes a distinct PAM, has a divergent target sequence length, and harbours different cutting characteristics. In tandem with the ease with which sgRNAs can be programmed, this extensive arsenal of CRISPR-based technologies now permits remarkable flexibility in gene editing experiments. Simultaneously, the simplicity and versatility of CRISPR/Cas editing systems has encouraged widespread use and displaced the need for less efficient, yet more arduous, systems, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and meganucleases [4, 5].

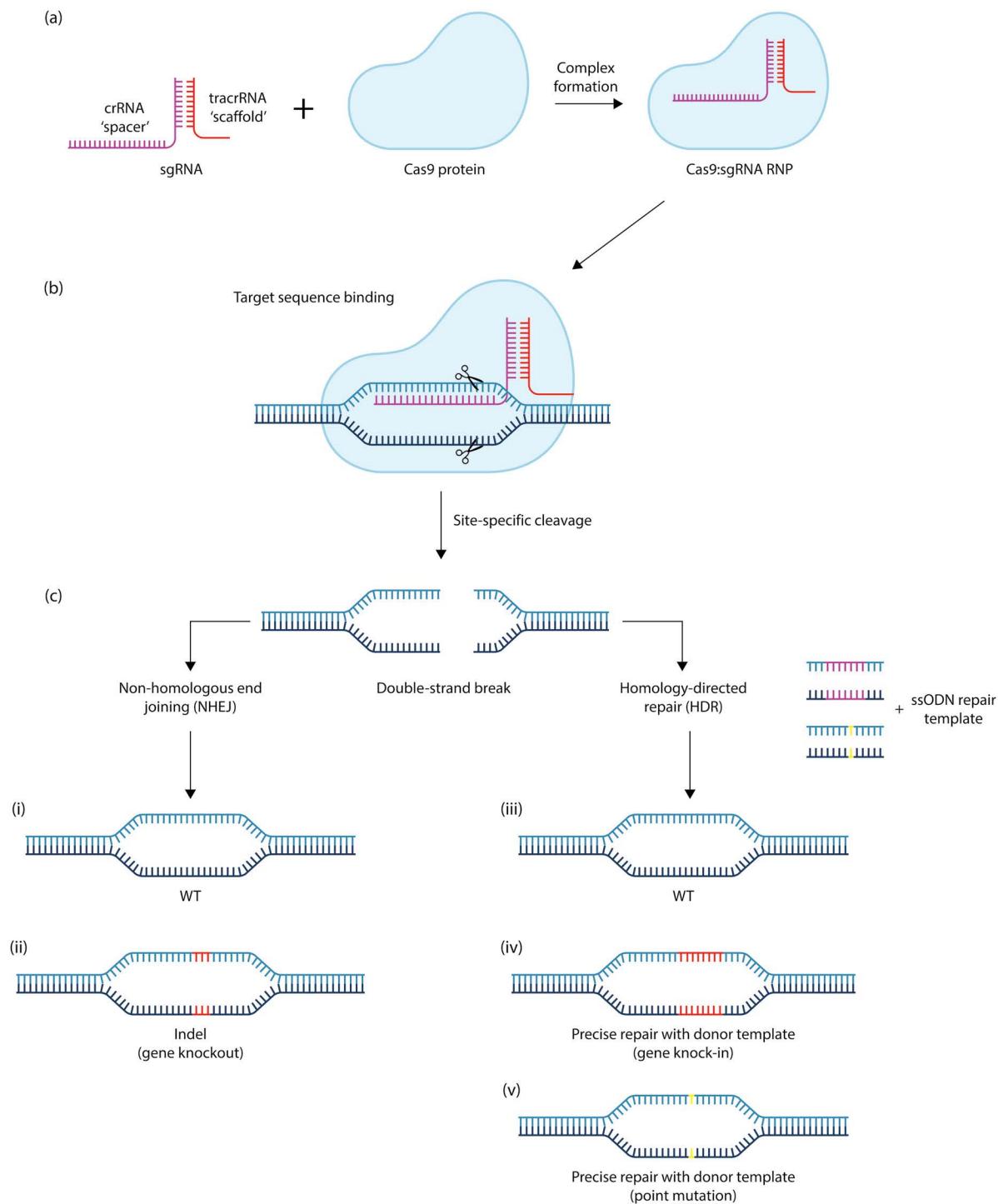


FIGURE 29.1 CRISPR/Cas-mediated DSB repair mechanisms. (a) The sgRNA consists of a “scaffold” trans-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA), which associates with a Cas protein to form an editing complex. (b) The crRNA “spacer” region directs the endonuclease to the target site. (c) Following the formation of a double-stranded break (DSB), endogenous DNA repair can occur by non-homologous end joining (NHEJ) resulting in (i) restoration of the Wt sequence, (ii) random indels that cause gene disruption or by homology-directed repair (HDR) which uses a template DNA strand for precise repair, resulting in (iii) restoration of Wt sequence, (iv) precise repair with gene insertion, or (v) precise repair with point mutation. Figure created using BioRender (www.biorender.com).

Assessing editing outcomes in CRISPR/Cas genome editing

Off-target editing and mosaicism are an ongoing challenge for CRISPR/Cas-mediated gene editing. Site-directed Cas endonucleases can unintentionally cleave host DNA at off-target sites, which can produce unwanted effects [23, 24]. Conversely, if editing complexes remain active throughout rounds of DNA replication, this can result in mosaicism: a phenomenon in which cells of the same organism carry distinct genotypes [12, 25]. Two studies attempting gene editing of 3PN human embryos both reported the occurrence of unintentional editing and mosaic mutants [26, 27]. Large chromosomal rearrangements and loss of heterozygosity have been highlighted as a further complications of CRISPR/Cas editing experiments [28–31].

Genome editing delivery strategies

There are three forms in which CRISPR/Cas gene editing systems can be introduced to target cells (Figure 29.2a) [21, 32]. The first strategy is to introduce a plasmid vector encoding both the Cas endonuclease and sgRNA [33]. Upon delivery into the cell, the *Cas* gene and sgRNA sequence are transcribed and the editing system is expressed until the plasmid is cleared from the cell (Figure 29.2b). However, failure of the host cell to efficiently clear plasmids can result in prolonged *Cas*/sgRNA expression and increase propensity for off-target editing [34]. Alternatively, editing components can be delivered as *Cas* mRNA and sgRNA, thus circumventing the use of host transcription. Yet, this delivery strategy invokes a “lag” time between *Cas* mRNA translation and sgRNA binding, which can increase incidence of mosaicism [35]. Finally, CRISPR/Cas systems can be introduced as ribonucleoproteins (RNPs). Here, the inherently functional Cas protein:sgRNA complex can begin genome editing instantaneously [27, 36]. In recent years, RNPs have become the dominant delivery strategy in CRISPR/Cas editing experiments. RNPs negate translation wait-time thus reducing mosaicism, whilst the shorter half-life of Cas proteins, compared to mRNA, diminishes off-target editing [27].

Methods for embryo transfection

Introduction of sufficient sgRNA and Cas endonuclease to target cells is fundamental to achieving high-impact editing outcomes. Delivery methods can be broadly classified into three groups: viral-based transduction, and chemical or physical transfection [37]. Transduction protocols are not compatible with germline editing due to high cytotoxicity, risk of viral infection, and potential integration of viral DNA into the host genome [38]. Conversely, chemical transfection methods, including liposomal and cationic polymer-based, have been trialled with limited success [39]. As such physical transfection techniques present the prevailing opportunity for introduction of CRISPR/Cas systems to zygotes. Microinjection persist as the predominant method for delivery of gene-editing components into the nucleus or cytoplasm of zygotes [39, 40]. Cytoplasmic injection is associated with higher embryo survival rates, as pronuclear injection can induce chromosomal breaks [41]. Yet, both types of microinjection are invasive and can pose serious harm to embryos, including post-transfection mortality [40]. Microinjection is a conceptually straightforward delivery method; however, necessitating a skilled technician to inject embryos individually, micromanipulation

techniques are accompanied by several practical challenges [42]. The manual requirement leaves the technique liable to major inter-operator variability, which can confound comparisons between experimental outcomes. To combat these limitations, the development of computer-assisted microinjection has permitted high-throughput micromanipulation of non-mammalian embryos with reproducible results [43]. So-called “autoinjection” has only recently been attempted in mammalian embryos and exhibited low mutational capacity [44, 45]. As such, the need for a safe, reproducible delivery method remains unabating.

Electroporation of embryos

Electroporation has emerged as a promising alternative to microinjection for zygotic transfection. The technique may be a uniquely beneficial transfection method for clinical applications: negating operator-specific variability, whilst simultaneously avoiding the risks associated with viral transduction [46]. Here, pulsed electric fields are employed to transiently increase cell membrane permeability, permitting entry of otherwise impermeant gene editing components into up to 100 embryos synchronously [47]. The capacity to transfect multiple zygotes simultaneously has significant advantages, allowing tight temporal regulation of delivery and augmenting consistency among samples. Conversely, sequential microinjection of embryos can result in sizable divergence in the time point at which CRISPR/Cas components are introduced to each embryo.

Electroporation is now routinely used to create transgenic mice. In these murine models, the technique has been shown to outperform microinjection: yielding higher mutation rates and bolstering offspring survival rates [48, 49]. Electroporation has also been successfully leveraged for CRISPR/Cas delivery to zygotes in larger mammals, with high-impact editing outcomes [50]. To our knowledge, electroporation-mediated delivery of CRISPR/Cas editing components is yet to be attempted on human embryos. Given the ease and reported successes of electroporation in animals, the application of electroporation in human germline editing experiments seems sensible. In preparation for this transition, it would prove worthwhile to investigate how electroporation could be deployed to enable more precise editing of human embryos.

Mammalian embryos—practical considerations

To date, research carried out on non-human embryos yields interesting but insufficient comparative evidence for understanding genome editing in human embryos. There are several practical considerations to evaluate regarding when introducing CRISPR/Cas systems to zygotes. Zygote size has been shown to affect gene editing outcome, as cell diameter is positively correlated with membrane potential [51]. As such, smaller embryos generally require a higher voltage to become permeabilized than larger embryos.

Mammalian oocytes are encapsulated by the zona pellucida: a thick, acellular, glycoprotein matrix that functions to support communication between oocytes and follicular cells during oogenesis and to protect oocytes and eggs during development [52, 53]. Bovine and porcine zonae pellucidae are both constituted of three glycoproteins (ZP1-3), whilst the human zona pellucida contains four distinct glycoproteins (ZP1-4) [54]. The human zona pellucida “hardens” upon fertilization to prevent

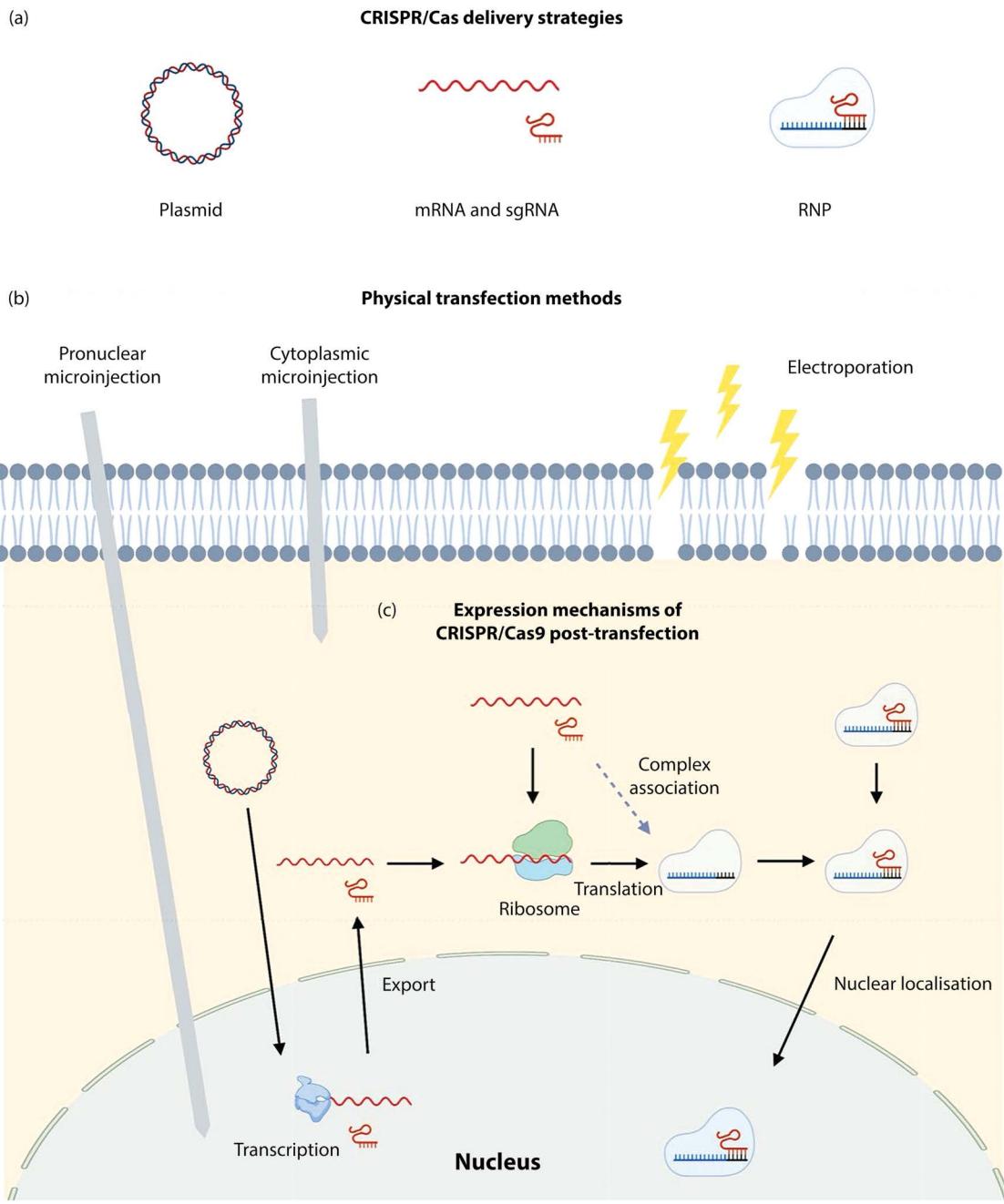


FIGURE 29.2 Strategies to deliver and edit genes using CRISPR/Cas9. (a) CRISPR/Cas systems can be introduced to cells in three forms: plasmid, mRNA, or RNP. (b) Pronuclear and cytoplasmic microinjection, as well as electroporation, are available physical transfection methods. (c) Plasmid delivery requires migration of the plasmid into the nucleus, transcription of the DNA, and exportation of the mRNA into the cytoplasm. The mRNA is then translated to produce Cas9 protein. The second strategy is to deliver a combination of the Cas 9 mRNA and the sgRNA. The Cas9 mRNA must be translated to Cas9 protein in cells from the Cas9/sgRNA complex. Finally, Cas9/sgRNA complexes can be delivered directly to cells. Figure created using BioRender (www.biorender.com).

polyspermy, conversely there is evidence to suggest bovine and porcine zonae pellucidae harden before fertilization [55, 56]. This “shell” may present a barrier to the delivery of editing components to zygotes and thus the zona pellucida warrants consideration in CRISPR/Cas editing experimental design. Early protocols for ZFN and TALEN-mediated editing typically reported zona

pellucida weakening by Tyrode’s solution prior to electroporation to improve editing efficiency [57]. Yet, loss of the zona pellucida may in turn affect embryo viability following electroporation [58].

Perhaps the most valuable use of genome editing will be in furthering our understanding of early human development, and the first licence was given to Niakan et al. to apply CRISPR/Cas9

to human embryos to interrogate the role of OCT4 in human embryo development [59].

Research has increasingly highlighted the marked mechanistic differences in embryonic genome activation (EGA), the initiation of gene expression following fertilization, between mice and humans [60, 61]. As such, whilst mice are undoubtedly an excellent model organism, they provide limited insights into human embryogenesis and present a poor candidate for elucidating how genome editing techniques can be best applied to human zygotes. In contrast, porcine, as well as bovine, embryos have been shown to better resemble human embryos (Figure 29.3) [62, 63]. Pigs also more closely model humans in terms of size, physiology, and genetics [64, 65]. As such, in a bid to advance safe and effective electroporation-mediated genome editing protocols for humans, efforts are perhaps better focused on larger mammalian embryos.

CRISPR/Cas gene editing has immense potential; however, several obstacles must be overcome before the technology can be widely deployed on human embryos. Our failure to establish delivery protocols that guarantee safe and efficient introduction of editing components to cells continues to present a significant challenge to widespread use.

Advancing tools in genome editing: Base editing and beyond

While genome editing strategies using nucleases hold great promise for the treatment of disorders, a major drawback of these traditional approaches is the generation of double-strand breaks

(DSBs), which can have unpredictable and potentially harmful effects. Base editing is a novel CRISPR-Cas9-based genome editing technology that allows the introduction of point mutations in the DNA without generating DSBs. This is achieved by using a specific type of enzymes, called Base Editors, which can change a single base by cutting one strand of the DNA and then using the cell's repair machinery to introduce a new base. Three major classes of base editors have been developed: Adenine Base Editors or ABEs, allowing A>G conversions, and Cytidine Base Editors or CBEs allowing C>T conversions [16]. The applicability and use of base editing tools has been extensively broadened to include Prime Editing (PE) [66], which can make more complex changes in the genome. This newer class of nucleases allows for greater specificity, higher efficiency, and increased accessibility to previously inaccessible genetic loci while maintaining a low rate of off-target effects as well as unwanted insertions and deletions. PE has expanded the CRISPR-base-edit toolkit to all 12 possible transition and transversion mutations, as well as small insertion or deletion mutations [67].

Base editing is a type of genome editing that allows for precise changes to a single base in the genome without making a double-stranded break (DSB) in the DNA. Base editing is a new and rapidly evolving field, which is becoming increasingly popular for its high precision and reduced off-target effects compared to traditional DSB-based genome editing methods such as CRISPR-Cas9. The high precision and reduced off-target effects of base editing make it a powerful tool for genetic research and have the potential to lead to new treatments and therapies for a wide range of genetic disorders.

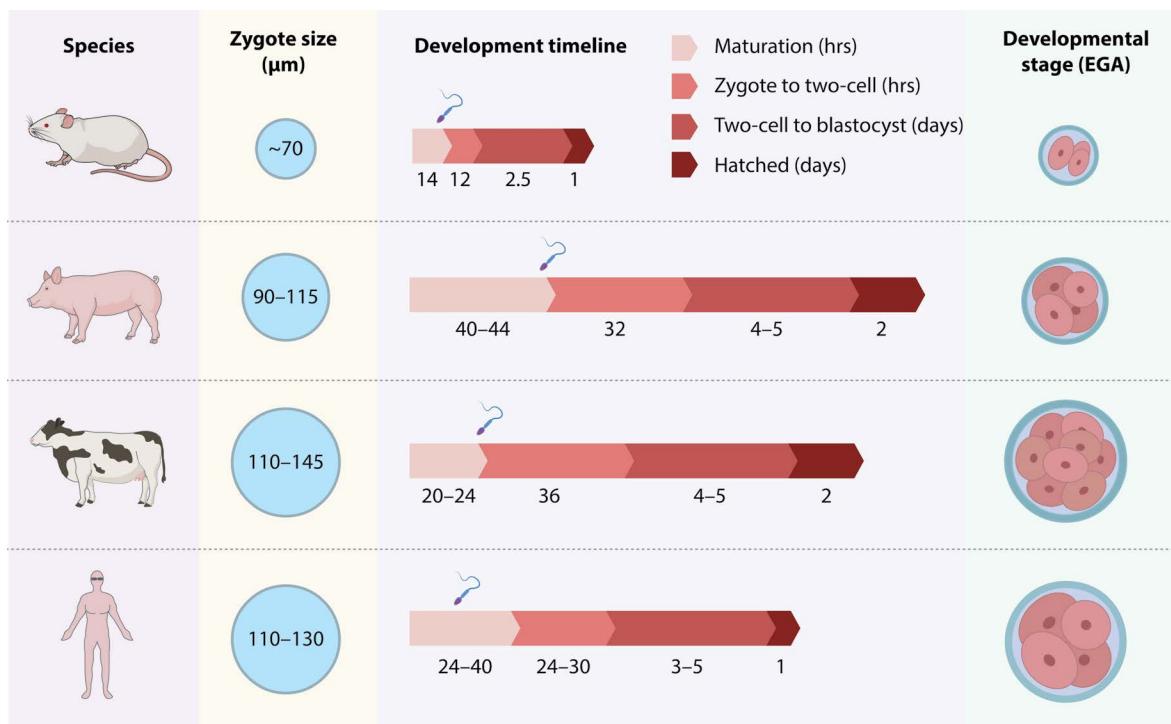


FIGURE 29.3 Oocyte size and timeline of early embryo development in mice, pigs, cattle, and humans. (From left to right): species; oocyte size (shown to scale for comparison); relative timeline of embryo development, including maturation, zygote to two-cell to blastocyst, then finally to hatching; and developmental stage at which EGA occurs. (Figure adapted under Creative Commons Attribution Licence from Santos RR, Schoevers EJ, Roelen BAJ, Usefulness of bovine and porcine IVM/IVF models for Reproductive toxicology, Reprod Biol Endocrinol. 2014; 12: 117; created using BioRender (www.biorender.com)).

Genome editing in human reproduction—past, present, and future

The 2018 International Summit on Human Genome Editing was a major international meeting held in Hong Kong in November 2018. The summit brought together leading experts from around the world to discuss the latest developments in genome editing and to consider the ethical, legal, and social implications of the technology. On the eve of the summit, a press release was made that Chinese scientist He Jiankui had used CRISPR-Cas9 genome editing to modify the genes of two human embryos.

The announcement by He Jiankui was met with widespread criticism from the scientific community, and the summit provided an opportunity for experts to address the concerns raised by his experiment [68, 69]. Many attendees expressed their disappointment with the lack of transparency and ethical considerations in He Jiankui's work, and they called for greater international collaboration and oversight in the development and use of genome editing technology.

The summit also served as an opportunity to discuss the potential benefits of genome editing, particularly in the area of human health. Attendees emphasized the need for responsible and ethical use of the technology, and they stressed the importance of continued research and development to ensure that the benefits of genome editing are realized in a safe and responsible manner.

In 2019, the World Health Organization (WHO) published a report on heritable genome editing, which called for a global ban on the use of CRISPR in human embryos [70]. The report emphasized the need for caution and transparency in the development and use of the technology, and called for international cooperation in developing ethical and regulatory frameworks to govern its use.

The Nuffield Council on Bioethics, a UK-based independent organization, also released a report on genome editing in the same year [71]. The report concluded that while the potential benefits of genome editing are significant, there are also significant ethical concerns, including the risk of creating new inequalities and the potential for unintended consequences. The report recommended that the use of CRISPR in human embryos be limited to cases where there is a serious medical need and that the technology be subject to rigorous ethical review.

In 2020, the National Academies of Sciences, Engineering, and Medicine in the United States released a consensus study report on genome editing [72]. The report agreed with the conclusions of WHO and Nuffield Council reports, and emphasized the need for caution in the development and use of the technology. The report called for rigorous ethical review of all proposals to use CRISPR in human embryos, and for the development of international guidelines to ensure that the technology is used responsibly.

Overall, the reports by WHO, Nuffield Council, and National Academies of Sciences, Engineering, and Medicine reflect the need for caution and transparency in the development and use of CRISPR technology, particularly in the area of human embryology. The reports emphasize the importance of rigorous ethical review and the development of international guidelines to ensure that the technology is used responsibly and in accordance with ethical standards.

In summary, genome editing is a revolutionary technology that has the potential to revolutionize medicine and biology by enabling the precise modification of genes. However, much

work is still needed to fully understand the breadth of its function, particularly in the context of human embryology and the ethical implications of editing the human germline. Despite the tremendous advances that have been made in the field, there are still many unknowns and uncertainties, and much research is needed to better understand the potential risks and benefits of this powerful technology. As the field of CRISPR genome editing continues to evolve, it is important that research is conducted in a responsible and ethical manner, taking into account the potential implications of these technologies for human health and well-being.

References

1. Sanson KR, Hanna RE, Hegde M, et al. Optimized libraries for CRISPR-Cas9 genetic screens with multiple modalities. *Nat Commun.* 2018;9:5416.
2. O'Neill HC. Clinical germline genome editing: When will good be good enough? *Perspect Biol Med.* 2020;63:101–110.
3. Jinek M, Chylinski K, Fonfara I, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science.* 2012;337(6096):816–21.
4. Cong L, Ran F, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science.* 2013;339(6121):819–23.
5. Mali P, Yang L, Esvelt K, et al. RNA-guided human genome engineering via Cas9. *Science.* 2013;339(6121):823–26.
6. Lu Y, Xue J, Deng T, et al. Safety and feasibility of CRISPR-edited t cells in patients with refractory non-small-cell lung cancer. *Nat Med.* 2020;26(5):732–40.
7. Stadtmauer EA, Fraietta JA, Davis MM, et al. CRISPR-engineered T cells in patients with refractory cancer. *Science.* 2020;367: eaba7365.
8. Lea R, Niakan K. Human germline genome editing. *Nat Cell Biol.* 2019;21(12):1479–1489.
9. Cyranoski D. The CRISPR-baby scandal: what's next for human gene-editing. *Nature.* 2019;566(7755):440–42. <https://www.nature.com/articles/d41586-019-00673-1> [Accessed 31 August 2021].
10. Brokowski C, Adli M. CRISPR ethics: Moral considerations for applications of a powerful tool. *J Mol Biol.* 2019;431(1):88–101.
11. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol.* 2018;36(8):765–771.
12. Mehravar M, Shirazi A, Nazari Met al. Mosaicism in CRISPR/Cas9-mediated genome editing. *Dev Biol.* 2019;445(2):156–62.
13. Hsu P, Lander E, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell.* 2014;157(6): 1262–78.
14. Rath D, Amlinger L, Rath A, et al. The CRISPR-Cas immune system: Biology, mechanisms and applications. *Biochimie.* 2015;117: 119–128.
15. Hille F, Charpentier E. CRISPR-Cas: Biology, mechanisms and relevance. *Philos Trans R Soc Lon B Biol Sci.* 2016;371(1707): 20150496.
16. Komor A, Badran A, Liu D. CRISPR-based technologies for the manipulation of eukaryotic genomes. *Cell.* 2017;169(3):559.
17. Mao Z, Bozzella M, Seluanov A, et al. Comparison of nonhomologous end joining and homologous recombination in human cells. *DNA Repair.* 2008;7(10):1765–71.
18. Van Den Bosch M, Lohman P, Pastink A. DNA double-strand break repair by homologous recombination. *Biol Chem.* 2002; 383(6):873–92.
19. Dudáš A, Chovanec M. DNA double-strand break repair by homologous recombination. *Mutat Res.* 2004;566(2):131–67.
20. Lin S, Staahl B, Alla R et al. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife.* 2014;3:e04766.

21. Liu C, Zhang L, Liu H, et al. Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. *J Control Release.* 2017;266:17–26.
22. Nakade S, Yamamoto T, Sakuma T. Cas9, Cpf1 and C2c1/2/3—What's next? *Bioengineered.* 2017;8(3):265–73.
23. Li J, Hong S, Chen W, et al. Advances in detecting and reducing off-target effects generated by CRISPR-mediated genome editing. *J Genet Genom.* 2019;46(11):513–21.
24. Zhang X, Tee L, Wang X, et al. Off-target effects in CRISPR/Cas9-mediated genome engineering. *Mol Ther Nucleic Acids.* 2015;4:e264.
25. Capalbo A, Ubaldi FM, Rienzi L, et al. Detecting mosaicism in trophectoderm biopsies: Current challenges and future possibilities. *Hum Reprod.* 2017 Mar 1;32(3):492–98. doi: [10.1093/humrep/dew250](https://doi.org/10.1093/humrep/dew250).
26. Kang X, He W, Huang Y, et al. Introducing precise genetic modifications into human 3PN embryos by CRISPR/Cas-mediated genome editing. *J Assist Reprod Genet.* 2016;33(5):581–88.
27. Liang P, Xu Y, Zhang X, et al. CRISPR/Cas9-mediated gene editing in human triploid zygotes. *Protein Cell.* 2015;6(5):363–72.
28. Cullot G, Boutin J, Toutain J, et al. CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. *Nat Commun.* 2019;10(1):1136.
29. Przewrocka J, Rowan A, Rosenthal R, et al. Unintended on-target chromosomal instability following CRISPR/Cas9 single gene targeting. *Ann Oncol.* 2020;31(9):1270–73.
30. Rayner E, Durin M, Thomas R, et al. CRISPR-Cas9 causes chromosomal instability and rearrangements in cancer cell lines, detectable by cytogenetic methods. *CRISPR J.* 2019;2(6):406–16.
31. Alanis-Lobato G, Zohren J, McCarthy A. Frequent loss of heterozygosity in CRISPR-Cas9-edited early human embryos. *Proc Natl Acad Sci U S A.* 2021;118(22):e2004832117. doi: [10.1073/pnas.2004832117](https://doi.org/10.1073/pnas.2004832117)
32. Yip B. Recent advances in CRISPR/Cas9 delivery strategies. *Biomolecules.* 2020;10(6):839.
33. Ran F, Hsu P, Lin C, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell.* 2013;154(6):1380–89.
34. Wu X, Kriz A, Sharp P. Target specificity of the CRISPR-Cas9 system. *Quant Biol.* 2014;2(2):59–70.
35. Hennig S, Owen J, Lin J, et al. Evaluation of mutation rates, mosaicism and off target mutations when injecting Cas9 mRNA or protein for genome editing of bovine embryos. *Sci Rep.* 2020;10(1):22309.
36. Kim S, Kim D, Cho S, et al. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* 2014;24(6):1012–19.
37. Lino C, Harper J, Carney J, et al. Delivering CRISPR: A review of the challenges and approaches. *Drug Delivery.* 2018;25(1):1234–57.
38. Robbins P, Tahara H, Ghivizzani S. Viral vectors for gene therapy. *Trends Biotechnol.* 1998;16(1):35–40.
39. Sato M, Ohtsuka M, Watanabe S, et al. Nucleic acids delivery methods for genome editing in zygotes and embryos: The old, the new, and the old-new. *Biol Direct.* 2016;11(1):16.
40. Horii T, Arai Y, Yamazaki M, et al. Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. *Sci Rep.* 2014;4(1):4513.
41. Yamauchi Y, Doe B, Ajduk A, et al. Genomic DNA damage in mouse Transgenesis. *Biol Reprod.* 2007;77(5):803–812.
42. Zhang Y. 2007. Microinjection technique and protocol to single cells. *Protocol Exchange.* <https://doi.org/10.1038/nprot.2007.487>
43. Wang W, Liu X, Gelinas D, et al. A fully automated robotic system for microinjection of zebrafish embryos. *PLoS ONE.* 2007;2(9):e862.
44. Doe B, Brown E, Boroviak K. Generating CRISPR/Cas9-derived mutant mice by zygote cytoplasmic injection using an automatic microinjector. *Methods Protoc.* 2018;1(1):5.
45. Eto T, Ueda H, Ito R, et al. Establishment of an integrated automated embryonic manipulation system for producing genetically modified mice. *Sci Rep.* 2021;11(1):11770.
46. Xu X, Gao D, Wang P, et al. Efficient homology-directed gene editing by CRISPR/Cas9 in human stem and primary cells using tube electroporation. *Sci Rep.* 2018;8(1):11649.
47. Kotnik T, Rems L, Tarek M, et al. Membrane electroporation and electroporation: Mechanisms and models. *Annu Rev Biophys.* 2019;48(1):63–91.
48. Miyasaka Y, Uno Y, Yoshimi K, et al. CLICK: One-step generation of conditional knockout mice. *BMC Genomics.* 2018;19(1):318.
49. Modzelewski A, Chen S, Willis B, et al. Efficient mouse genome engineering by CRISPR-EZ technology. *Nat Protoc.* 2018;13(6):1253–74.
50. McFarlane G, Salvesen H, Sternberg A, et al. On-farm livestock genome editing using cutting edge reproductive technologies. *Front Sustain Food Syst.* 2019;3.
51. Agarwal A, Zudans I, Weber E, et al. Effect of cell size and shape on single-cell electroporation. *Anal Chem.* 2007;79(10):3589–96.
52. Modliński J. The role of the zona pellucida in the development of mouse eggs in vivo. *Development.* 1970;23(3):539–47.
53. Bleil J, Wassarman P. Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zona pellucidae possessing receptor activity for sperm. *Cell.* 1980;20(3):873–82.
54. Moros-Nicolás C, Chevret P, Jiménez-Movilla M, et al. New insights into the mammalian egg zona pellucida. *Int J Mol Sci.* 2021;22(6):3276.
55. Fahrenkamp E, Algarra B, Jovine L. Mammalian egg coat modifications and the block to polyspermy. *Mol Reprod Dev.* 2020;87(3):326–340.
56. Coy P, Canovas S, Mondejar I, et al. Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proc Natl Acad Sci U S A.* 2008;105(41):15809–14.
57. Grabarek J, Plusa B, Glover D, et al. Efficient delivery of dsRNA into zona-enclosed mouse oocytes and preimplantation embryos by electroporation. *Genesis.* 2002;32(4):269–76.
58. Hakim B, Tyagi V, Agnihotri S, et al. Electroporation of mouse follicles, oocytes and embryos without manipulating zona pellucida. *J Dev Biol.* 2021;9(2):13.
59. Fogarty NM, McCarthy A, Snijders KE, et al. Genome editing reveals a role for oct4 in human embryogenesis. *Nature.* 2017;550:67–73.
60. Niakan K, Eggan K. Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse. *Dev Biol.* 2013;375(1):54–64.
61. Li L, Lu X, Dean J. The maternal to zygotic transition in mammals. *Mol Aspects Med.* 2013;34(5):919–38.
62. Jukam D, Shariati S, Skotheim J. Zygotic genome activation in vertebrates. *Dev Cell.* 2017;42(4):316–32.
63. Sansom KR, Hanna RE, Hegde M, et al. Optimized libraries for CRISPR-Cas9 genetic screens with multiple modalities. *Nat Commun.* 2018;9:5416. doi: <https://doi.org/10.1038/s41467-018-07901-8>
64. Whyte J, Prather R. Genetic modifications of pigs for medicine and agriculture. *Mol Reprod Dev.* 2011;78(10–11):879–91.
65. Walters E, Wells K, Bryda E, et al. Swine models, genomic tools and services to enhance our understanding of human health and diseases. *Lab Animal.* 2017;46(4):167–72.
66. Anzalone A, Koblan L, Liu D. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat Biotechnol.* 2020;38(7):824–44.
67. Kantor A, McClements ME, MacLaren RE. CRISPR-Cas9 DNA base-editing and prime-editing. *Int J Mol Sci.* 2020 Aug 28;21(17):6240. doi: <https://doi.org/10.3390/ijms21176240>.

68. O'Neill HC, Cohen J. Live births following genome editing in human embryos: A call for clarity, self-control and regulation. *Reprod Biomed Online*. 2019;38(2):131–132. doi: <https://doi.org/10.1016/j.rbmo.2018.12.003>.
69. Lovell-Badge R. CRISPR babies: A view from the centre of the storm. *Development*. 2019 February1;146(3):dev175778. doi: <https://doi.org/10.1242/dev.175778>.
70. Baylis F, Darnovsky M, Hasson K, et al. Human germline and heritable genome editing: The global policy landscape. *CRISPR J*. 2020;3:365–377.
71. Nuffield Council on Bioethics. *Genome Editing and Human Reproduction: Social and Ethical Issues*. London: Nuffield Council on Bioethics, 2018.
72. National Academy of Sciences. *Heritable Human Genome Editing*. Washington, DC: The National Academies Press, 2020. <https://doi.org/10.17226/25665>.

30

DESIGNING DISASTER PLANS FOR IVF LABORATORIES

Kimball O. Pomeroy and Michael L. Reed

Not all disasters are as dramatic as a hurricane ravaging an entire city, a forest fire threatening a clinic, or an earthquake tearing apart a hospital. There are many unsuspecting disasters that come upon us without warning. A laboratory flooded in Boston when a water line broke on the fifth floor and 28,000 gallons of water flooded the downstairs. An IVF clinic in San Diego, although not threatened by nearby fires, had toxic air around the clinic for several weeks potentially affecting outcomes in IVF cycles. In Arizona, a toilet valve stuck, flooding the laboratory with two inches of water. In New Zealand, an IVF clinic below a birthing facility was inundated with water from a tub left on above them. After trying many methods to remediate the resulting mould growing everywhere in the laboratory, they finally had success in using hydrogen peroxide mist to fumigate the building. Some, like those located in Florida and southern Texas are accustomed to activating disaster plans every year due to the common occurrence of hurricanes in their areas. We can learn from these examples and be better prepared.

Dr Richard Dickey, who was involved with hurricane Katrina's impact on the Fertility Institute of New Orleans reported, "Our experience with Katrina proved that it is not necessary to be at ground zero to be affected by a natural disaster. An event miles away may disrupt electrical power and accessibility for an extended period of time; therefore, having to rely on generators until power is restored and delaying transfer to the fifth day may not be sufficient. All IVF programs need plans to protect fresh and cryopreserved embryos in the event of a natural or human-made disaster" [1].

No matter where a laboratory is located, even if it is not in a hurricane or earthquake zone, there are many catastrophes that can ruin a clinic and its patients' chances of a healthy birth. The recent Covid-19 pandemic has emphasized how important disaster plans are. These plans should be made in a time of relative safety when one has time to thoroughly think through how to best get through an emergency. In many cases, resources may already be, or will become, scarce and the first person, if prepared, can react to these disasters to secure these resources. Better yet, these resources can be secured before the disaster occurs.

In the beginning of the Covid-19 pandemic, most people were left on their own to react while waiting for the experts to release responses. Nobody in the fertility industry foresaw a pandemic as a cause for concern, certainly not enough concern to enact a pandemic preparedness plan, despite the warnings from HIV and ZIKA.

"The primary objective of an emergency action plan (the 'Emergency Plan') should be to provide for the safety of program personnel and patients, fresh and cryopreserved human specimens, and critical equipment and records" [2].

Types of disasters

Disasters can be divided into two major categories, natural and man-made. Natural disasters include hurricanes, tornados, forest fires, floods, earthquakes, blizzards, and high winds. Man-made threats include terrorism, arsonists, and strikes.

The consequences of these disasters must be considered for each potential disaster. Flooding may occur from a backed-up toilet or from a river that overran its banks. Electrical outages can occur from too much demand on the power system or from a hurricane. Shortages of materials can occur from a workforce stoppage, strike, or from a pandemic.

In developing a plan, all potential interruptions and sources of damage to the laboratory and clinic should be considered. Each type of disaster may necessitate a different solution to the specific type of damage, depending on available resources. Flooding from a backed-up toilet can be overseen by a flood recovery company, but these companies most likely will be overburdened after a hurricane; and so a separate solution must be found—maybe purchasing your own clean-up equipment including vacuums and pumps prior to an emergency.

Regulations pertaining to disaster plans

The laboratory and clinic have both a regulatory and an ethical responsibility to its patients and its staff during a disaster situation. These responsibilities include protection (people and tissue); continuation of services when possible; and a rapid, safe, and responsible recovery from the dangers imposed by the disaster. A comprehensive disaster plan, if done correctly, can make sure these responsibilities are taken care of.

Laboratories and clinics are required by regulatory bodies such as the College of American Pathologists (CAP), the American Society of Reproductive Medicine, and the European Society of Human Reproduction and Embrology to have disaster preparedness plans to help them in handling emergencies [3–6]. The CAP checklist item Gen.73.800 addresses disaster plans and states, "The specific elements to be included in the emergency preparedness plan must be based on a risk assessment using an 'all-hazards' approach to evaluate the types of hazards most likely to occur that would potentially disrupt services" [3]. The Joint Commission standard EM.09.09.01 requires an "all-hazard" approach to the development of a comprehensive disaster preparedness plan [7]. It addresses leadership roles, communication during the emergency, staffing during the emergency, a disaster recovery plan, and an emergency education plan. These plans must ensure the safety of staff, patients, and tissue.

Software to aid in making a disaster plan

There are several software programs (Noggin Emergency Management, for example) that advertise management solutions, disaster planning, incident reporting, prediction algorithms, and so forth, but be aware that many are focused on large geographic disaster scenarios. Most ART programs have specific needs, and you may spend time and money trying to modify or force a program to meet your needs. If you are a member of a larger group or corporate institution, a software option may already be available to you, so make sure to check with a member of your institution's safety committee. Also, many of these programs are aimed at data recovery, and are less useful for general planning purposes. If you are not part of a larger institution, a colleague may be willing to share their policies and procedures and could be adapted as needed.

How to design a disaster plan

Designing a disaster plan is not easy. There are several tools available to aid in their development. One of the best resources is from the Federal Emergency Management Agency (FEMA). They provide numerous "kits" that will walk you through the development of plans for several types of disasters. These kits are available free at their website <http://www.ready.gov/business>. There are kits to help design plans for power outages, flooding, high winds, earthquakes, and hurricanes.

The American Red Cross has a free, self-guided program to help businesses, organizations, and schools become prepared for emergencies (<https://www.readyrating.org/How-It-Works>) [8]. This program, Ready Rating, consists of five sections: participation, emergency planning, facility and equipment, training and exercises, and extended community. These programs will guide one through best industry practices in making and implementing a preparedness plan. A group's preparedness is rated by answering questions in either the ReadyGo or the ReadyAdvance module. The ReadyAdvance program will probably fit the needs of most fertility practices. It will measure an emergency preparedness plan's maturity and completeness. The Red Cross also has several free phone apps that address many types of emergencies. These applications can generate alerts for areas that are of interest to you—your home, the clinic, your loved ones—when an emergency is approaching. They also have information on how to prepare and what to do and not do during a particular emergency.

Getting started

The best way to get started, if you do not already have a plan to work from, is to outline and address topics, then revisit areas of concern with other team members. Initially you will no doubt generate more questions than answers. See Box 30.1.

Create a safety team if you have not already done so. Include physicians or anaesthetists participating in anaesthesia for egg retrievals or other office surgical procedures, as evacuations may require ambulatory measures. Also include nurses, medical assistants, and clerical staff in the design of a disaster plan. Assign specific tasks, and make sure to follow up to be sure that all tasks are being addressed. Chemical, biological, and environmental safety are all areas to consider.

The initial key part of any disaster plan is the investigation phase. The investigation phase should include all departments involved—the laboratory, clinical staff, and office staff. In this phase, one should brainstorm and write down the most common

BOX 30.1 STEPS TO DISASTER PLANNING

1. Take photos of the entire laboratory and clinic.
2. Identify key equipment.
3. Build a disaster committee that includes staff members from every department.
4. Start with addressing disasters/emergencies that have occurred in the past and then look at the rarer ones in order of probability of occurring.
5. List important individual functions that might be disrupted.
6. Design protocols for how to manage important individual functions and at varying levels of interruption.
7. Visualize the disaster and what items need to be cared for.
8. Itemize protocols to mitigate these items of concern prior to the disaster.
9. Itemize protocols that will be implemented during the disaster. Be sure to include when they will be implemented, how they will be implemented, and who will implement them.
10. Address communication concerns for a major disaster.
11. Include in the protocols what will happen when the disaster is either minimized or is gone.
12. Design a method to train the staff on what their part will be in the disaster.

emergencies that one can foresee. Examine the last 10 years and include these emergencies first. Ideally, one may want to start by listing specific mechanical emergencies and use these plans to work into more general emergencies. These mechanical emergencies might include loss of power, loss of water, loss of HVAC, general flooding, stoppage of supplies, evacuation of a building, inability to enter the laboratory, etc. The general emergencies might include flooding, smoke, earthquake, hurricane, and pandemic, for example. Focus on one plan at a time. Even if it is not possible to think of every potential emergency, these mechanical emergencies can form the basis for assisting in any emergency. It might help in the next steps, and especially in the recovery phase, to take a wide-angle photo of each room in the clinic and make a list of equipment and key supplies. Not only will these photos help in planning, but they will also assist if insurance is needed to corroborate recovery costs. A full inventory of any assets in the clinic should be done. This can also be used for insurance purposes during the recovery phase.

Good preparation for a disaster includes the design of informed consents so that patients will have been told prior to an emergency what will potentially happen to their treatment and their tissues. Included in this could be an alternative method of communication with the clinic that clinic employees can monitor. Ideally, this should be a site where the patient can leave a question and the staff can monitor daily.

When making a plan, set aside time, without distractions, for a walk-through of all spaces. Have copies of the floorplans available for your own space and areas outside of your space. Make sure to take notes on all ingress and egress points and have team members open and close doors. Also, not all doors open in the same direction. Some may be one-way doors that lock when they close behind you, or have magnetic or powered properties, and

alarms. A walk-through will also ensure that the space on the other side of an egress door is not blocked or cluttered or used for storage—you don't want anything to block the exit and there may not be time to clear a path. If your facility has windows, inspect these as well—can they be used as an escape route, or are they a hazard in high winds? Are you in a facility with multiple floors? If so, make sure that you have ready access to stairwells—again, can the doors be opened?—and don't forget to walk the stairwells and see where they lead. You might be surprised, and you don't want to end up in a dead-end courtyard or parking garage with few or blocked exits.

One of the prominent components of an overall disaster plan is the evacuation route—fire, earthquakes, power outages, and many other scenarios involve the safe evacuation of staff and patients. This is the most likely part of the disaster plan that will be activated, as most emergencies on a larger or smaller scale will require that you move from your current location to a designated meeting site.

Consider how well your evacuation route will work for ambulatory situations. Can you move freely carrying someone, moving someone in a wheelchair or on a gurney? Look for obvious engineering controls, e.g. fire extinguishers (you may have to use them to clear a path through a fire), emergency lighting, emergency exit signs that come on during any power failure.

Purchase flashlights for the clinic if you do not already have them. A real example—the clinic was in the basement of a physicians' office building, with no windows or outside natural lighting. The power lines to the building were cut by a backhoe, including a nearby power line from the emergency backup generator. No light, no power, pitch black, with patients and staff still in the clinic. A few individuals had cell phones and were able to use them as flashlights while being evacuated.

Find a safe meeting point that everyone is familiar with—keep it simple and use obvious landmarks, visible in low light or darkness, and in low visibility conditions.

Once you have set up an evacuation route, assign specific duties to individuals to clear the workspace. Bathrooms, offices, break rooms, exam rooms, closets, and storage rooms—don't miss a single place. Make sure that everyone knows their areas of responsibility. Better yet, if you have enough staff, team up to help each other and patients.

Mitigation

The next step of the plan is mitigation [9]. In mitigation, one should focus on what can be done to either avoid the disaster or to decrease the harm from a disaster. For example, in case of minor flooding this might include making sure all electrical equipment is raised above the surface of the floor to avoid minor flooding and destruction of critical electrical equipment.

One fertility clinic had minor flooding from an overflowing toilet. Had the tower computer been placed on a small platform to raise it just two inches from the floor, a vital piece of equipment would not have had to be taken out of service until a new computer could be put into service. Another mitigation to minor flooding could be installation of drains in critical areas and water monitors/alarms to provide early warning of water encroachment. Approaches should be taken to not only reduce harm but also to avoid harm if possible.

For each type of potential disaster, examine what can be done to decrease the disaster's affect. How can electrical equipment be protected from electrical surges? How can windows be protected from damage due to high winds? Should storm shutters

be installed? How can critical items be protected from high heat? If the HVAC goes down, how will incubators function? Will an alternative portable refrigeration unit be needed? Do you have enough liquid nitrogen for a lock out of a week? How will preparation for potential shortages in the supply chain of materials be managed? What quantity of important laboratory and clinical supplies will be kept on hand? Will a secondary source of information of clinical records or cryo-storage records be available if needed and local computers and the internet are down? One should sit down with the disaster planning team to come up with a brain-storming list of all potential problems that might occur.

Response phase

The response phase is next; and it is a major part of any disaster plan. It describes what will happen when a disaster hits and how the effects will be minimized with proper backups, contingencies, and sufficient supplies. In this phase, it is important to decide what items will be needed to handle the emergency when it occurs so that they can be purchased ahead of time. For example, if one needs to move cryo-storage tanks, are there wheeled carts or hand trucks available to move them? If the elevators are not functioning, is there a system to allow for the relocation of equipment and tissue that does not rely on the elevator? How will staff keep in contact if cellular phone systems are inoperable?

One needs to plan what the reactions should be to the emergency. Who will instigate the plan and under what circumstances? How will storage tanks be moved and where will they be moved to? If a fire in a nearby forest is close, what will the response be? What responses will occur when a hurricane watch is posted for your area? What about when a hurricane warning is posted? How will current or upcoming IVF cases be managed?

An IVF group in Miami bases their hurricane disaster plan on when a hurricane is several days out (Inea Collazo, personal communication). At this time, they sit down and chart out all of the patients that have embryos in the incubator and those that are in stimulation. When a watch is declared, which means the hurricane is about two days away, they begin vitrifying all embryos in the incubators and all patients that are in the middle of a stimulation are cancelled.

An effective way to approach preparing a disaster plan is to first design plans for each type/level and duration of interruption see **Box 30.2**. These can be called sub-disasters. For example, a hurricane disaster may include several sub-disasters such as flooding, power outages, and disruption of transportation. Identify these sub-disasters and design plans for differing levels of these sub-disasters which can then be plugged into a major disaster

BOX 30.2 SUB-DISASTERS TO ADDRESS IN PLANNING

1. Power outages
2. Flooding
3. Inability to access the clinic
4. High winds
5. Too hot or cold
6. Toxic air
7. Supply chain stoppage or slow down
8. Communication interruptions
9. Transportation interruptions

plan. Consider flooding. Design responses for minor flooding and major flooding, e.g. for floods lasting hours and for floods lasting days. Then look at power outages that are short-term versus long-term. Have a plan for no physical access to the facilities for a day versus many days. These plans for each type of interruption can then be added to each disaster plan depending on the type of disaster.

Even with practice, expect that someone will panic during a real disaster—routine drills will help alleviate this, but there is no guarantee that everyone will stay calm. Having to control a panicked person is difficult—emotionally and physically. Panic can be contagious. During a real evacuation, one may experience fire, smoke, loud alarms, flashing lights, loud voices, and sounds—it could feel dark, chaotic, frightening, and there is no way to predict how others could react. Some individuals may be terrified, afraid to move forward or backward until physically guided out of danger, whereas others might run without looking back. So be prepared to take charge of the situation. If you find yourself panicking, get help if you can, and try not to get separated from the group.

Recovery phase

The final phase of writing a disaster plan is the recovery phase. It examines what will happen after the disaster happens and how the laboratory or clinic will resume operation. The first priorities should include ensuring safety, providing essential needs, and restoring basic services. Have a contact list for local and state agencies, e.g. fire, police, state police, as they will be able to help determine if, and when, you can return. What criteria must be met to reopen and how will one ensure that the laboratory is safe and ready to culture and freeze embryos? How will debris be removed? How will clinical and laboratory areas be cleaned? What steps will be taken to ensure the incubators and other critical equipment are working correctly?

When the disaster has passed, and it has been deemed safe to enter the building, it is important to do a complete inventory of damage. It is at this time that professional disaster recovery companies can be invited in to help evaluate and offer suggestions regarding the building's structural integrity, safety as relates to utilities, and options to remove debris and restore the working space to its original. If flooding has occurred, special attention should be placed on how to mitigate contamination of the building from fungi.

First attempts to mitigate damage do not always work and so one must monitor the outcomes of recovery attempts over time until success is achieved. A clinic in Australia was inundated with water when a jacuzzi in another office space upstairs flooded the downstairs IVF clinic. At first, a simple vacuuming of the water and clean up was done, but after a few weeks, it became apparent that fungi from the walls was contaminating the IVF cultures. Several methods were tried to kill the fungi, but none were successful until hydrogen peroxide mist was used to fumigate the entire clinic.

A video camera and/or a camera can be used to record the aftermath. Notes should be taken on the damage to the facility and the equipment. The initial inventory of equipment may be useful for this, especially if some of the equipment is missing. Attention should be paid to functionality and safety.

Power outages

Power outages are common in many parts of the world. How you prepare for them depends on how long these outages typically

last. The most common method used for short-term outages is a battery backup. Chains of batteries may work well for an hour or two (depending on the equipment one is backing up) but are not a solution for all but minor outages. Batteries often are heavy and so are placed on floors where inundating water may damage them. They can be protected from minor flooding by placing them on raised platforms or on shelves. It is important that only critical equipment, such as incubators and refrigerators are drawing current during an emergency. Providing enough batteries for powering all the equipment for an egg retrieval during an emergency may be excessive due to the current drawn from ultrasounds and warmers.

An on-site generator is the next level of protection. Generators can run on gasoline or natural gas. They can also be purchased and installed so that they will automatically come on should the power go out. There are several problems with generators though. One, is their cost. Two, is the amount of maintenance and testing required. Finally, if a big disaster hits, will there be fuel available to run these generators for several days? Storing of fuels and the generator with its toxic fumes is another problem. Natural gas is probably a better fuel source than gasoline, as natural gas sources are often operable even during major disasters while dependence on transportation may decrease the availability of gasoline during a major disaster. In some emergencies, one may be able to depend on the rental of large mobile generators. In most emergencies that last several days, most likely all procedures will be halted, and so long-term power may not be needed once embryos are cryopreserved. For this reason, cryo-storage methods that are static and require no power are ideal. Still, one must consider how cryo-storage alarms will be managed in each type of emergency. The inclusion of solar arrays for disaster recovery should also be considered.

During any power outage, concerns regarding the heating or cooling of the building, maintenance of refrigerators and freezers, and any current embryos in incubators need to be addressed. For example, all incubators will lose the ability to pump gasses, but depending on the make/model, temperature may be maintained for a longer, or brief, time. All power backup systems should be tested periodically for efficacy and how long they can provide backup. If batteries cannot provide adequate time for the maintenance of equipment, they need to be replaced.

Communication during a disaster

A system for communication during a disaster is one of the most often overlooked items. Communication is critical; how will you communicate during and after evacuation? What will you do if someone is not accounted for after an evacuation? Going back for someone may not be safe—so make sure to communicate effectively, and determine where everyone should be, where everyone is. If land line or cell phones are not working, how will the coordination of the disaster plan occur? How will you know proper steps have been taken to ensure the safety of tissues and equipment?

In 2011, a tornado struck the area around Tuscaloosa, Alabama. It caused 54 fatalities, 1500 injuries, and even more were left homeless. Druid City Hospital was the sole remaining building in the centre of destruction. As a result of problems during the disaster, the hospital began to evaluate push-to-talk devices as a method to improve communication [10].

It is important to have a central location (and possibly an alternate one) for communication during the disaster. One IVF clinic has a phone line with an answering machine that can be used as a

central messaging centre during a disaster. Some communication methods may not be available during distinct parts of a disaster. For example, initially, power may be out so that one must depend solely on methods that do not use the local electric grid. Also, during the initial part of any major disaster, local mobile services are often either unavailable or so clogged with traffic that communication using cell phones is impossible. Have an awareness of who is in the clinic, staff, and patients. Sign in boards for staff and patients can help and prevent leaving someone behind.

Forms of communication that should be considered are cell phones, texting, fax machines, email, ham, or citizen band radios. Instantaneous communication may not be available in some circumstances and so a passive system where one can leave a message for later retrieval may be best. Texting is a good method for communicating when cell service is overloaded or erratic.

Prior to any emergency, employees should all have access to phone numbers for employees, service staff, current patients, stakeholders, suppliers, and key incident response personnel (governmental and private disaster agencies, for example). The time to put together this extensive list is prior to the disaster. This list can be secured in a centralized system or location. In most major disasters it will be important to have access to patient medical charts to advise on patients undergoing treatment. Access to cryopreservation records will also be important as patients call with questions on their frozen specimens.

There should be a day-by-day plan prior, during, and after a disaster for all procedures in progress, either a whiteboard, an electronic or paper sheet for each incubator explaining what must be done each day. These worksheets can be used to make decisions—what procedures need to be done today, what will need to be done tomorrow, for example, retrievals, inseminations, fertilization checks, hatching, biopsy, freezing, and so on. These lists can be used by staff in the event of personnel shortages, immediate or anticipated, to bring additional staff into the laboratory the same or next day—the lists will help bring the auxiliary staff up to speed on what is required.

Transportation

Soon after hurricane Katrina hit the New Orleans area in 2005, the laboratory director of a local IVF lab, Roman Pyrzak, was able to obtain satellite images from NASA that showed him his hospital was surrounded by water but was intact. He reached out to several agencies to help him evacuate the cryo-storage tanks using boats [11]. These groups included the National Guard and local police. Lack of transportation can occur due to many causes, including fuel shortages, bridge outages, road closures, evacuations, traffic jams, and the stopping of public transportation. Disaster plans should address how to manage these circumstances. Consider using those that live closest for handling some of the critical steps of the plan if transportation becomes an issue.

Training

Development of a disaster plan is a futile exercise if nobody knows what their job will be should a disaster occur. Periodic training is key to the execution of a good plan. In this training,

staff should know when to act and how to act. They should be assigned tasks for each disaster type. Because these plans may be full of what-ifs and can be complex, it is important that each staff member can easily access the plan from work and home so that they can remind themselves of what their role is in the disaster plan. Assign someone to document the drills, not just doing it, but every aspect—when the drill started, the people involved, and how they behave. Review the effectiveness of drills at least annually—if something is not working, then adapt the plan.

It may be important to have refresher courses just prior to a critical time when a disaster has the highest probability of occurring. For example, just prior to hurricane or fire season. After each training session, it is an excellent time to ask for input from the trainees on how the plan can be improved. Having periodic drills for specific disaster scenarios should be considered.

References

1. Dickey RP, Lu PY, Sartor BM, Dunaway HE Jr., Pyrzak R, Klumpp AM. Steps taken to protect and rescue cryopreserved embryos during hurricane Katrina. *Fertil Steril*. 2006;86:732–4.
2. Development of an emergency plan for in vitro fertilization programs: A committee opinion. Practice Committees of the American Society for Reproductive Medicine, the Society for Assisted Reproductive Technology, and the Society of Reproductive Biologists and Technologists. *Fertil Steril*. 2021;115(4):870–3.
3. CAP Laboratory General Checklist 9/22/21. College of American Pathologists; 2021.
4. Revised guidelines for human embryology and andrology laboratories. *Fertil Steril*. 2008;90(5):S45–59.
5. The Revised guidelines for good practice in IVF laboratories (2015) [Internet]. [cited 2022 Jan 2]. Available from: [https://www.esre.eu/Guidelines-and-Legal/Guidelines/Revised-guidelines-for-good-practice-in-IVF-laboratories-\(2015\).aspx](https://www.esre.eu/Guidelines-and-Legal/Guidelines/Revised-guidelines-for-good-practice-in-IVF-laboratories-(2015).aspx).
6. Practice Committee of the American Society for Reproductive Medicine, Practice Committee of the Society for Assisted Reproductive Technology, Practice Committee of the Society of Reproductive Biologists and Technologists. Minimum standards for practices offering assisted reproductive technologies: A committee opinion. *Fertil Steril*. 2021;115(3):578–582.
7. The Joint Commission. R3 Report: Requirement, Rational, Reference [Internet]. 2021 [cited 2022 Feb 25]. Available from: <https://www.jointcommission.org/standards/r3-report/>.
8. American Red Cross Ready Rating. American Red Cross Ready Rating [Internet]. 2022 [cited 2022 Feb 9]. Available from: <https://www.readyrating.org/How-It-Works>.
9. National Research Council. Prudent Practices in the Laboratory: Handling and Management of Chemical Hazards, Updated Version [Internet]. Washington, DC: The National Academies Press; 2011 [cited 2021 Dec 30]. 360 p. Available from: <https://www.nap.edu/catalog/12654/prudent-practices-in-the-laboratory-handling-and-management-of-chemical>.
10. Scungio DJ. Disaster preparedness plans [Internet]. Medical Laboratory Observer. 2014 [cited 2021 Nov 6]. Available from: <https://www.mlo-online.com/home/article/13006800/disaster-and-the-laboratory-preparation-response-and-recovery>.
11. Roman P. Crises Management Symposium. ASRM Scientific Congress and Exposition 2021; 2021 Oct 20; Baltimore, Maryland.

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EARLY HUMAN EMBRYO DEVELOPMENT REVEALED BY STATIC IMAGING

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Introduction

Our understanding of early human embryo development has for many decades derived from examining images of fixed specimens and, only more recently, cultured human embryos. Careful analysis of serial sections of human embryos retrieved during hysterectomies and stained with haematoxylin and eosin revealed the complex morphological changes the embryo must undergo as it transits through the fallopian tube and implants into the uterus [1]. For many years these scarce specimens provided nearly all our information about early human development. However, the subsequent development of *in vitro* systems that enable human embryo culture, combined with improvements in microscopy technologies constitutes a major advance that has allowed a glimpse into early embryogenesis and even the previously intractable process of implantation. Human embryos can develop through early to late implantation stages in 2D co-cultures with supporting cells, or in more complex 3D models respectively [2]. It is now also possible for embryos to undergo attachment *in vitro* without any exogenous cells or tissues present, enabling the investigation of embryo-autonomous peri-implantation organization [3–5]. Imaging *in vitro*-implanted embryos at various stages is revealing novel insights into processes directing embryo development and implantation. Innovative imaging technologies and new automated analysis approaches offer the potential to non-invasively select embryos with the best developmental capacity. Visualizing the molecular and morphological changes that occur as the preimplantation embryo develops is essential to drive new knowledge to improve the outcomes of assisted reproductive technologies (ART).

Human embryo development

Day 1: Zygote

Fertilization of an oocyte by a sperm to form a zygote triggers a series of morphological changes, including extrusion of the second polar body and the development and gradual migration of the male and female pronuclei. Once formed, the female pronucleus migrates towards the male pronucleus until they are in apposition and move together to the centre of the oocyte. Inside the migrating pronuclei, nuclear precursor bodies appear and coalesce into nucleoli. For development to proceed, these events—which depend on both maternal and paternal factors—must be executed in a timely and coordinated manner.

Various systems for grading zygotes have been developed based on several easily visible parameters including the number, size, and distribution of nucleoli and the size and alignment of pronuclei [6–8]. Scores assigned on these features likely reflect the fidelity of critical early processes such as chromosomal segregation, decondensation of chromatin, and activation of zygotic RNA synthesis, and have been associated with the developmental potential of the embryo [7, 9]. However, the highly dynamic

nature of these features makes the scoring very sensitive to the timepoint at which they are measured, perhaps explaining why many studies do not support an association between zygotic scoring and pregnancy [10–13].

During the first few days of human development, the fertilized zygote undergoes a series of cleavage divisions in which each cell splits in half, producing two smaller cells (or blastomeres) without changing the total volume of the embryo. These divisions are prone to errors due to the high rate of chromosomal instability in human embryos [14]. Confocal microscopy of fixed human embryos donated from clinical *in vitro* fertilization (IVF) cycles showed that even good quality embryos have many nuclear abnormalities, including cells with small additional micronuclei, cells with two equally sized nuclei, and cells with many small nuclei (Figure 31.1) [15].

Cells with abnormal nuclei are likely to be aneuploid and show evidence of DNA damage. However, the frequency of nuclear abnormalities decreases as development progresses, suggesting a potential error-correcting mechanism may exist. Although the underlying causes of these errors in chromosome segregation are not known, it has recently been demonstrated that disruption of the mitotic spindle in human cleavage-stage embryos does not trigger cell death as would normally occur in somatic cells (16).

Day 2: Four-cell stage

The second cleavage division produces an embryo with four blastomeres. Depending on the cleavage orientation of each blastomere at the 2-cell stage, the 4-cell embryo may have either a tetrahedral or planar configuration (Figure 31.2).

Given that at least some proteins display a polarized localization in the oocyte, the orientation of the cleavage divisions can result in unequal distribution of these proteins between blastomeres from the 4-cell stage onwards [18]. It is currently unknown what determines the orientation of the cleavage divisions; however, tetrahedral 4-cell embryos are more likely to develop into high-quality embryos *in vitro* [17, 19].

Day 3–4: Morula Compaction

The first obvious morphogenetic process to occur during pre-implantation development is compaction. During compaction the cells of the embryo flatten against each other, increasing their contact areas and transforming the embryo from a loose cluster of spherical cells into a tightly packed mass [20]. Compaction may be initiated between the 4- to 16-cell stages, but most human embryos begin to compact at the 8-cell stage and the timing of compaction is associated with blastocyst quality and implantation [21, 22]. The cell adhesion molecule, E-CADHERIN accumulates at cell-cell contacts in compacting embryos and abnormal distribution of E-CADHERIN is associated with developmental defects including non-compaction, cell fragmentation, and embryo arrest [23].

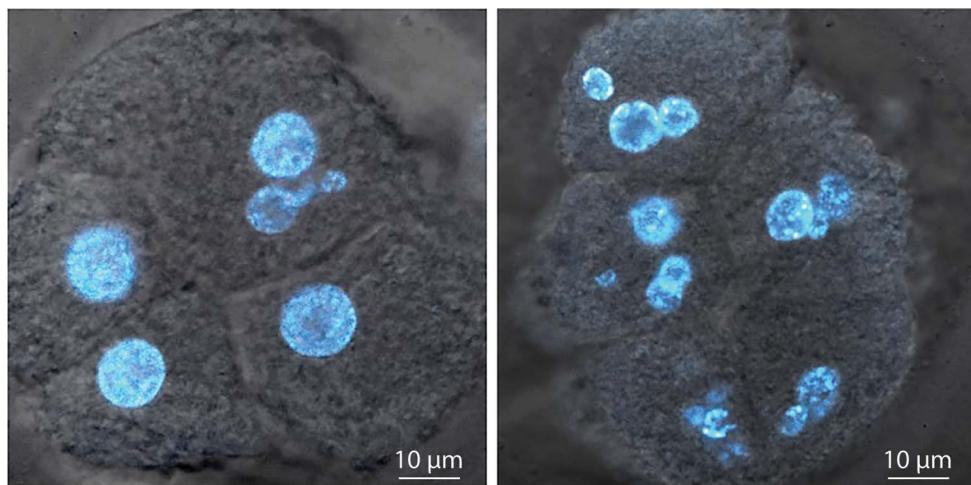


FIGURE 31.1 Nuclear abnormalities in human cleavage stage embryos. (Adapted with permission from [15].)

Polarization

Concurrent with compaction in human embryos is the establishment of apical-basal cell polarity. Immunofluorescence and confocal microscopy of human embryos fixed on day 4 of development revealed that the apical domain is generated in two steps [24]. First, activation of Phospholipase C (PLC) signalling triggers the enrichment of actin at the contact-free apical surface of the blastomeres. Next, the PAR polarity complex components PARD6 and aPKC are localized to the nascent apical domain [24, 25]. Establishing the apical domain promotes onset of the first cell lineage differentiation: a trophectoderm-associated transcriptional program characterized by expression of the GATA3 transcription factor and nuclear localization of YAP1 (Figure 31.3). The trophectoderm (TE) mediates implantation of the embryo into the uterine wall and will give rise to the placenta. Inhibiting PLC or aPKC signalling impairs the initiation of TE specification and the expansion of the first cavity to form a blastocyst [24, 25].

Day 5–6: Blastocyst

The human pre-implantation blastocyst is a hollow ball of cells consisting of a small cluster of tightly packed pluripotent cells

(the inner cell mass, ICM) located at one side of the cavity, surrounded by larger TE cells forming the external surface. These first two cell lineages can be distinguished based on differential expression of transcription factors (Figure 31.4).

ICM cells express OCT4 and either NANOG or variable levels of GATA6 and will give rise to the embryonic tissues [4]. The extraembryonic TE cells express GATA3, variable levels of CDX2, and low levels of OCT4 and GATA6. Once the blastocyst has fully expanded, it will hatch from the zona pellucida and is ready for implantation. The recent development of culture systems that facilitate *in vitro* attachment of human embryos is enabling the first in-depth investigations of peri-implantation development and revealing critical processes driving this previously intractable developmental stage (Figure 31.5).

Day 7–8: Epiblast and hypoblast segregation

Failure of the embryo to implant into the uterus is a major cause of early pregnancy loss and a critical barrier that must be overcome for successful ART [26, 27]. Implantation requires the blastocyst to adhere to the epithelial layer of the endometrium and invade into the endometrial stroma under the epithelium. Here it

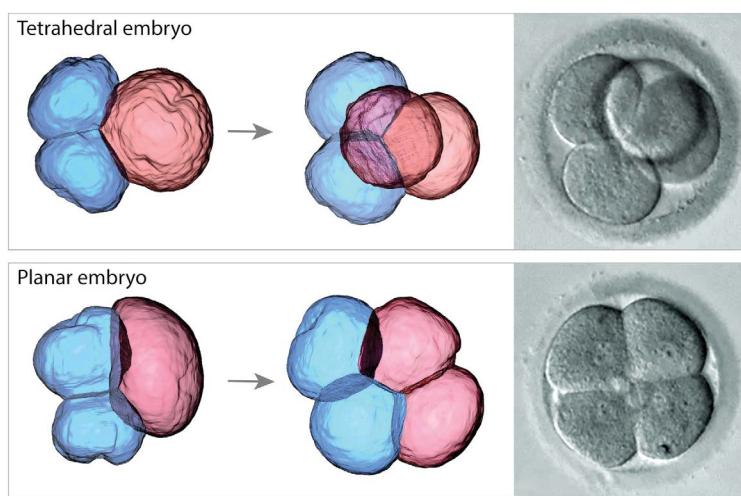


FIGURE 31.2 Different cleavage orientations produce tetrahedral or planar embryos. Images of human 4-cell stage embryos on day 2 of pre-implantation development. (Adapted with permission from [17].)

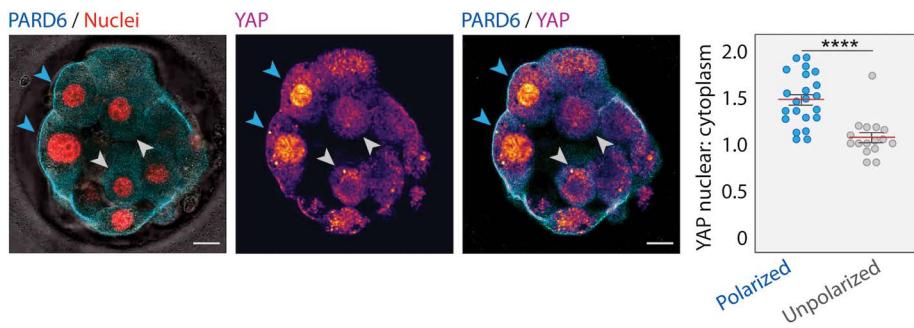


FIGURE 31.3 Human embryos establish apical-basal polarity on day 4. Embryos immunolabelled with PARD6 and YAP1 and stained with DAPI to label nuclei. Blue arrowheads indicate outer cells, white arrowheads indicate inner cells. Graph shows quantification of the nucleus to cytoplasm ratio of YAP1. ***p < 0.0001, Mann–Whitney test. (Adapted from [24] under Open Access.)

must penetrate and remodel maternal blood vessels to establish a blood flow for subsequent development.

Addition of blastocysts to a relatively simple 2D co-culture system comprised of a monolayer of Ishikawa cells derived from an endometrial adenocarcinoma demonstrated that most, if not all, good-quality embryos will initiate attachment with receptive luminal epithelial cells [28]. The majority of human embryos attach at the polar TE, which is the region of TE that surrounds the ICM [4, 28–30]. This initial attachment is likely mediated by cell adhesion molecules shown to be upregulated in the human blastocyst such as integrin $\alpha v\beta 3$, thrombospondin 1, and laminin $\alpha 3$ [28, 31].

As the embryo undergoes attachment, it flattens and begins a cellular reorganization that segregates the embryonic and extraembryonic cells [3, 4]. The embryonic epiblast cells are

characterized by OCT4 expression and will give rise to the fetus. On days 7–8, the epiblast consists of a cluster of OCT4-positive cells surrounded by GATA6-positive extra-embryonic hypoblast cells (primitive endoderm), which will form the yolk sac (Figure 31.5). *In vitro* culture of embryos in atmospheric air (21% O₂) conditions demonstrated improved preservation of epiblast cells and reduced cell death [3]. However, in IVF clinics, human pre-implantation embryos are increasingly cultured in hypoxic conditions (5% O₂) as this replicates the oxygen concentration in the oviduct and uterus and is proposed to favour embryo survival [32, 33]. Given these conflicting findings, it is of utmost importance that the molecular effects of oxygen concentration on epiblast development and embryo survival be carefully evaluated as this a parameter that may be easily controlled for the improvement of IVF outcomes.

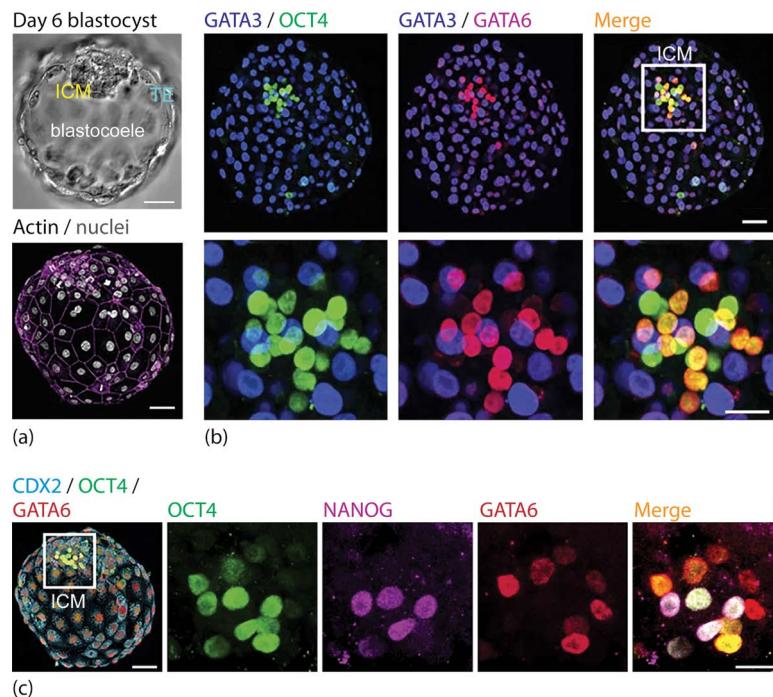


FIGURE 31.4 Establishment of the first two cell lineages in the day 6 human embryo. (a) DIC image (above) and 3D projection of day 6 blastocyst stained with Phalloidin to label actin and DAPI to label nuclei (below). Scale bar 100 μ m. (b) and (c) 3D projections of day 6 blastocysts immunolabelled with markers for ICM and TE. (Adapted with permission from [4].)

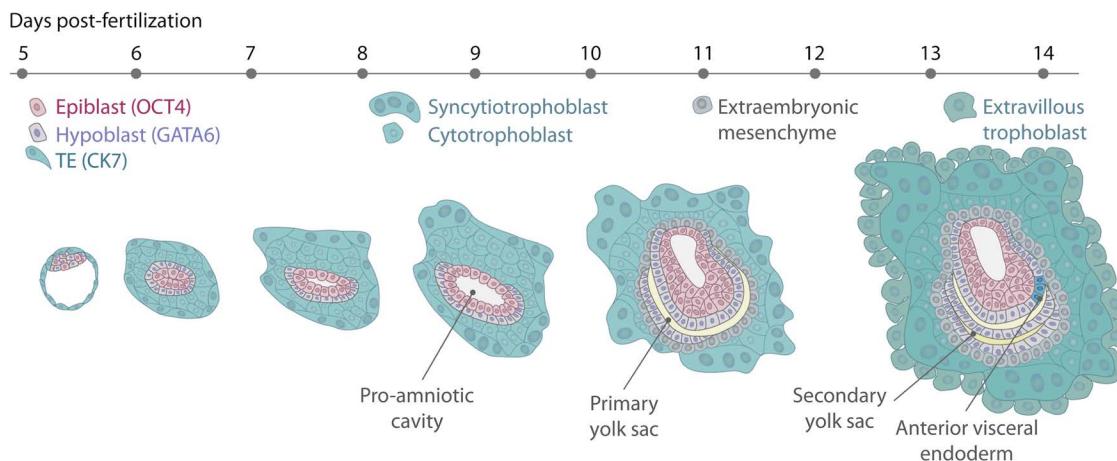


FIGURE 31.5 Schematic of *in vitro* implanted human embryo development.

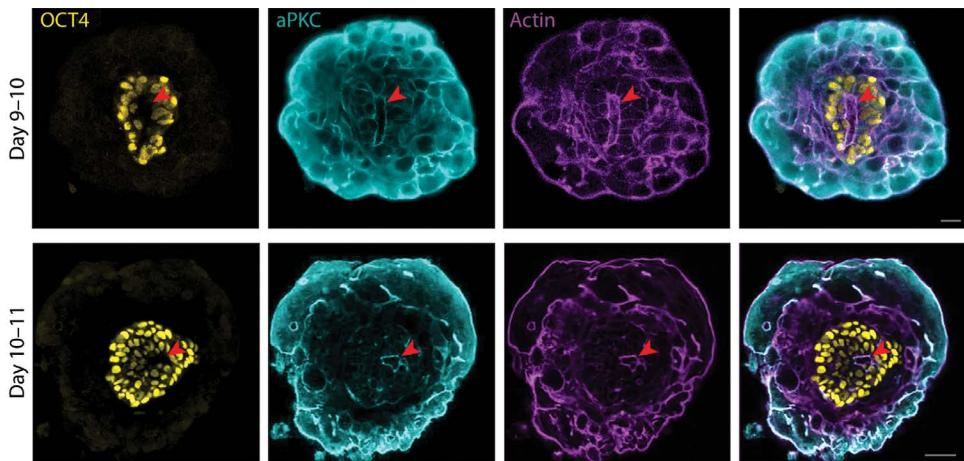


FIGURE 31.6 Formation of the pro-amniotic cavity in cultured human embryos. Confocal Z-sections through the centre of human embryos at different stages immunolabelled for OCT4 and aPKC and stained with Phalloidin to label actin. The arrowhead indicates the presence of a lumen. Scale bar, 20 μ m. (Adapted with permission from [3].)

Day 8–10: Epiblast polarization and formation of pro-amniotic cavity and primary yolk sac

From days 8 to 10 *in vitro*, the OCT4-expressing epiblast cells become radially organized and establish apical-basal polarity [3, 5]. Actomyosin and the main kinase of the apical PAR polarity complex, aPKC, become progressively restricted to the apical domain of the cells, whereas integrin β 1 is confined to the baso-lateral domain [3, 34]. A subset of the epiblast cells displays apical localization of the lumenogenesis component, PODXL, and a small lumen lined with actin cytoskeleton forms, indicating the onset of pro-amniotic cavity formation (Figure 31.6) [4, 35].

Concurrently, on days 8–9 the hypoblast cells become localized to one side of the epiblast and an increasingly restricted subset of cells express CER1 and LEFTY1, antagonists of WNT, BMP and NODAL signalling pathways [3, 5, 36]. This population of cells may serve as a signalling centre to initiate patterning of the anterior-posterior axis of the embryo before gastrulation. Adjacent to the hypoblast cells, the putative yolk sac cavity forms (Figure 31.7).

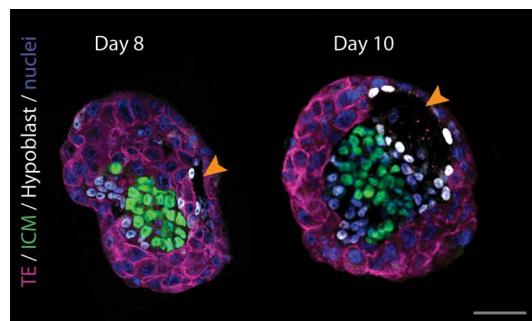


FIGURE 31.7 Development of the primary yolk sac from day 8–10 in a human blastocyst. Confocal Z-section through the middle of an embryo immunolabelled for ICM (OCT4, green), hypoblast (GATA6, grey) and TE (CK7, magenta). Arrowhead indicates developing primary yolk sac. Scale bar, 50 μ m. (Adapted with permission from [5].)

and is lined by a novel cell type expressing CDX2 and low levels of GATA6 and OCT4, recently described as yolk sac TE [4].

Trophoblast differentiation

On the surface of the embryo, the TE cells are polarized with apical localization of PAR6, cytokeratin 7 (CK7), and actin [3, 5]. Beginning at day 8, two subpopulations of TE cells emerge (Figure 31.8). TE cells closest to the epiblast and hypoblast retain a single nucleus and likely correspond to the cytотrophoblast (CTB) lineage. However, TE cells in the periphery of the embryo begin to express human chorionic gonadotropin (HCG) and fuse to become multinucleated, a feature of the syncytiotrophoblast (STB) lineage (Figure 31.8a) [3, 4]. Expression of HCG is important for the formation of the STB, modulation of endometrial

receptivity at the implantation site and subsequent development of umbilical circulation [37]. Low levels of HCG are associated with recurrent miscarriage and non-viable pregnancies following ART [38, 39], demonstrating the vital role of trophoblast differentiation and sufficient HCG expression in establishing and maintaining pregnancy.

Recent work studying whole chromosome aneuploidies in *in vitro* cultured human embryos revealed that embryos with trisomy 16 or monosomy 21 are smaller due to underdevelopment of their trophoblast cells (Figure 31.8c) [41]. In trisomy 16 embryos, the trophoblast hypo-proliferation was proposed to result from overexpression of the E-CADHERIN gene, which is located on chromosome 16 and promotes cell cycle arrest and trophoblast differentiation. Indeed, immunofluorescence and confocal

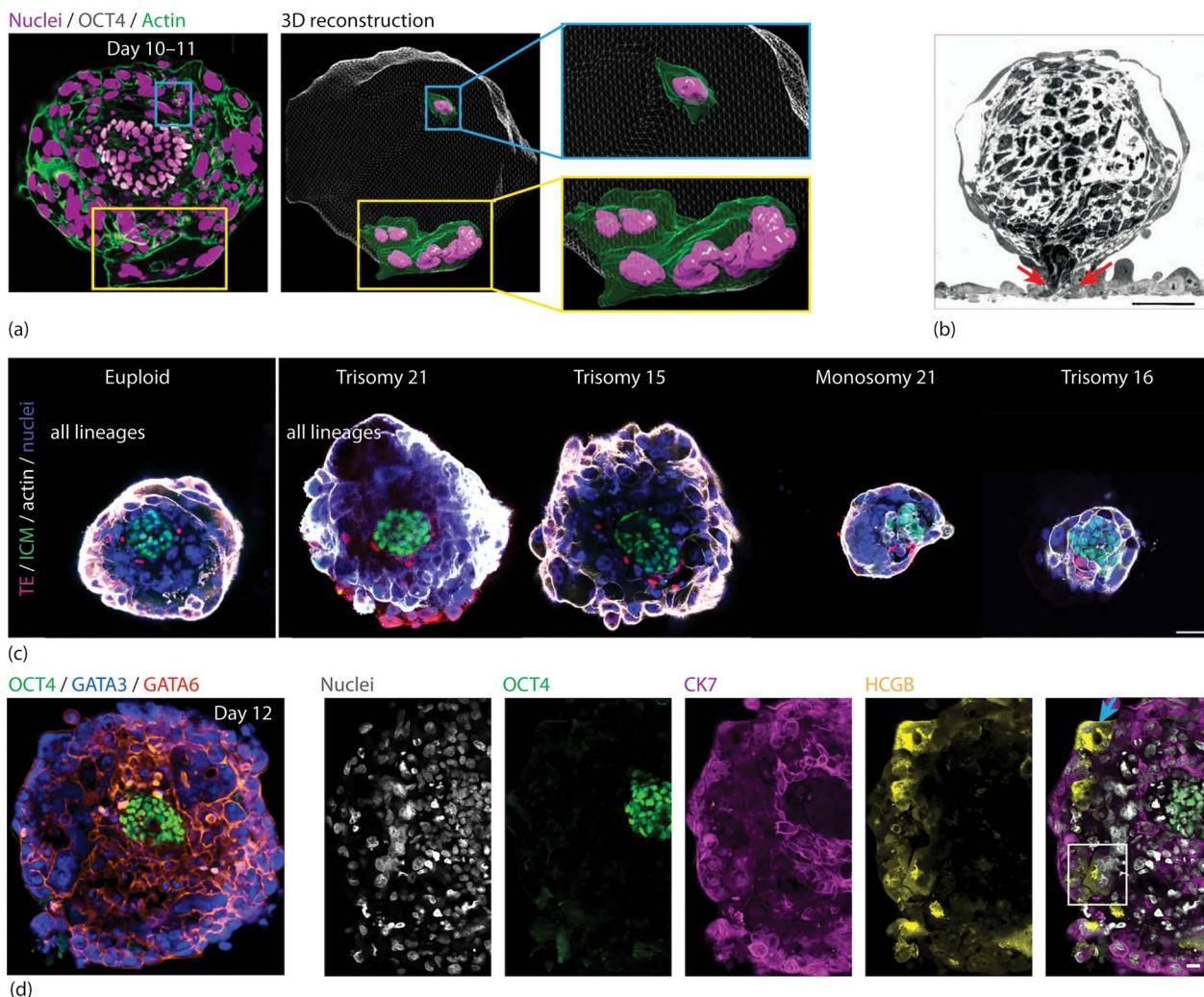


FIGURE 31.8 Trophoblast differentiation in implanting human embryos. (a) 3D reconstruction of the cellular and nuclear shape of representative trophectoderm cells. Note that cells near the epiblast have a single nucleus, whereas cells in the periphery of the embryo are multinucleated. (b) Light micrograph of human blastocyst adhering to cultured endometrial cells and forming a trophoblastic penetration cone (arrows). The penetration cone is occupied by cells from the inner cell mass that almost fill the blastocyst cavity. (c) Day 9 human embryos with whole chromosome aneuploidies. Embryos are immunolabelled to show TE (GATA6, magenta) and ICM (OCT4, green) and stained for actin (Phalloidin, grey) and nuclei (DAPI, blue). Monosomy 21 and trisomy 16 embryos are small due to a lack of trophoblast cells. (d) Day 12 human blastocyst showing differentiation of syncytiotrophoblast (CK7, HCGB) lineage. The blue arrow indicates an example of nascent lacuna, typical of STB cells; the box indicates an example of multinucleated cells characteristic of STB lineage progression. Scale bar, 20 µm. ([a] Adapted with permission from [3]; [b] Adapted with permission from [40]; [c] Adapted from [4] under Open Access; [d] Adapted with permission from [4].)

imaging of trisomy 16 embryos demonstrated increased levels of E-CADHERIN protein and the trophoblast differentiation marker SDC-1, combined with lower numbers of mitotic trophoblast cells. These findings suggest a potential explanation for the intrauterine growth restriction and pre-eclampsia often observed in cases of placental trisomy 16 [41].

At the implantation site, the STB cells are highly invasive, forming contacts with uterine endometrial cells and pushing in between them [1, 29, 40, 42]. As more STB cells penetrate the endometrial layer, they can form a cone which contains fully surrounded epiblast cells (Figure 31.8b) [40]. The endothelial cells are displaced and accumulate around the implantation site. Invasion of the trophoblast cells is proposed to exert pulling and stretching forces on the epiblast, which may contribute to its transformation into a bilaminar disc-like structure at this stage [43].

The STB is also characterized by the formation of lacunae: interconnected spaces that will contain maternal blood once the STB cells breach the maternal vessels (Figure 31.8d) [29, 44]. This lays the foundation for the maternal-utero circulatory system, which will eventually supply the fetus with sufficient blood flow to support development through the second and third trimesters. The CTB and STB cells continue to reorganize into concentric rings from days 9 to 12 until the STB forms the outer trophoblast layer and the CTB is restricted to the areas adjacent to the epiblast and developing yolk sac [3, 4, 44].

Day 10–14

Careful analysis of *in vitro*-attached blastocysts revealed that from day 10, the epiblast cells appear to segregate into two sub-populations [3]. Epiblast cells adjacent to the hypoblast acquire a columnar morphology, express E-CADHERIN adhesion proteins at the cell membrane, and become the bilaminar epiblast disc [3, 5]. However, the epiblast cells which are in contact with the CTB become flat and squamous, likely representing the prospective amniotic epithelium (AME). Expansion of the amniotic cavity separates the epiblast disc from the AME [5]. By day 11 of development, the primary yolk sac is fully formed [1, 3, 5].

In vitro 3D culture of human embryos up to the 14-day limit established by internationally recognized guidelines [45] allowed the development of the secondary yolk sac and the initiation of gene expression that defines the anterior-posterior axis of the embryo (LEFTY) and early hallmarks of the primitive streak (N-CADHERIN and T, Figure 31.9) [5].

Remarkably, the human embryo can direct all these lineage specification, cellular reorganization, and early tissue morphogenesis events autonomously without input from any maternal tissues [3, 4]. *In vitro* culture systems that support early human development are enabling an unprecedented view of these early processes, and the insights they yield offer hope for the improvement of embryo culture and selection for ART.

Alternative microscopy approaches for non-invasive embryo imaging

The clinical benefits to both mother and child of transferring a single embryo during IVF are now commonly accepted [46–48], placing increasing importance on the ability to select the embryo with the most developmental potential for transfer. Traditionally, embryos have been selected based on their morphological features, making microscopy an indispensable tool for ART [49]. Despite the recent advent of time-lapse incubation systems which take digital images at regular intervals to enable continuous monitoring of embryo development, static morphological assessment remains the standard approach for embryo selection worldwide. Typically, embryologists remove the embryo from the incubator at specific timepoints and examine it on a microscope. A score is assigned based on morphological features such as blastomere number, fragmentation and symmetry and the quality of the ICM and TE [50]. Although continuous embryo monitoring enables morphokinetic analysis, the higher cost of time-lapse incubation systems remains a significant barrier in many IVF clinics and it is not yet clear whether the technology improves implantation rates [51, 52].

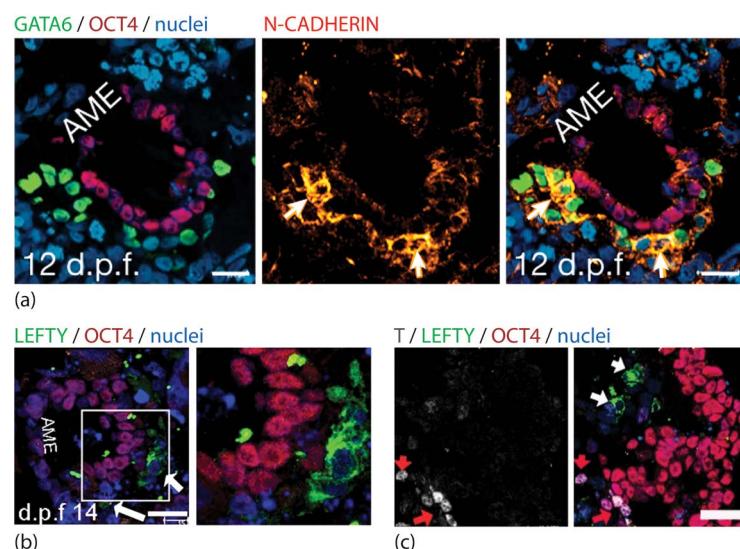


FIGURE 31.9 Expression of primitive streak markers and anterior-posterior polarity in human embryos from days 12–14. (a) Immunolabelling of day 12 embryo for ICM (OCT4, red), hypoblast (GATA6, green), and mesenchymal marker (N-CADHERIN, orange). (b) and (c) Anterior marker LEFTY (green) and an early primitive streak marker (T, grey) are expressed in a day-14 embryo. Abbreviation: AME, amniotic epithelium. Scale bar, 50 μ m. (Adapted with permission from [5]).

Polarization microscopy

Standard laboratory microscopes are equipped with brightfield illumination which is excellent for visualizing stained specimens but resolves few details in unstained live cells. To improve imaging of live specimens, modern microscopes often have specialized objectives and condensers, or light polarizers to provide phase contrast that allows far greater resolution of subcellular features. Polarization microscopy has been of particular use in embryology due to its ability to significantly increase the image quality of thick unlabelled specimens (Figure 31.10) [53, 54]. This technique images macromolecular structures based on their birefringence; a unique optical property whereby light entering a sample containing highly ordered molecules is refracted as two light components with differing phase. Birefringence materials are characterized by having two orthogonal optical axes, with a different index of refraction along each axis. Light beams parallel to one of the optical axes travel at a different speed through the sample than does light polarized parallel to the orthogonal axis. As a result, these two light components, which were in phase before they entered the sample, are retarded, and exit the sample out of phase. Measuring this differential retardation quantifies the magnitude and orientation of molecular order in the specimen.

Polarizing microscopy made it possible to image the mitotic spindles in unlabelled living cells due to the array of aligned spindle microtubules (Figure 31.10) [56–58]. Subsequent improvements include adding electro-optical modulators, employing circularly polarized light, and exploiting the angle dependence of birefringence to visualize other cytoskeletal elements in living cells, including stress fibres and vesicular structures travelling along the cytoskeleton [60, 62, 65, 69].

Two structures in the mammalian egg that exhibit molecular order when imaged with polarized optics are the meiotic spindle [63] and zona pellucida [61]. Prior to the introduction of polarization microscopy, it was extremely difficult to visualize the spindle in live human oocytes. Using polarization microscopy, it became possible to examine spindle dynamics, detect spindle morphology, predict chromosome misalignment, monitor thermal control, and perform spindle transfer [70–73]. Numerous studies have investigated whether the presence of a spindle in human oocytes is associated with improved ART outcomes. Although the results of these studies are sometimes contradictory (reviewed in [74]), a meta-analysis of 10 trials determined that oocytes with a spindle detectable by polarization microscopy show higher rates of fertilization and faster rates of cleavage and embryo development up to the blastocyst stage [75].

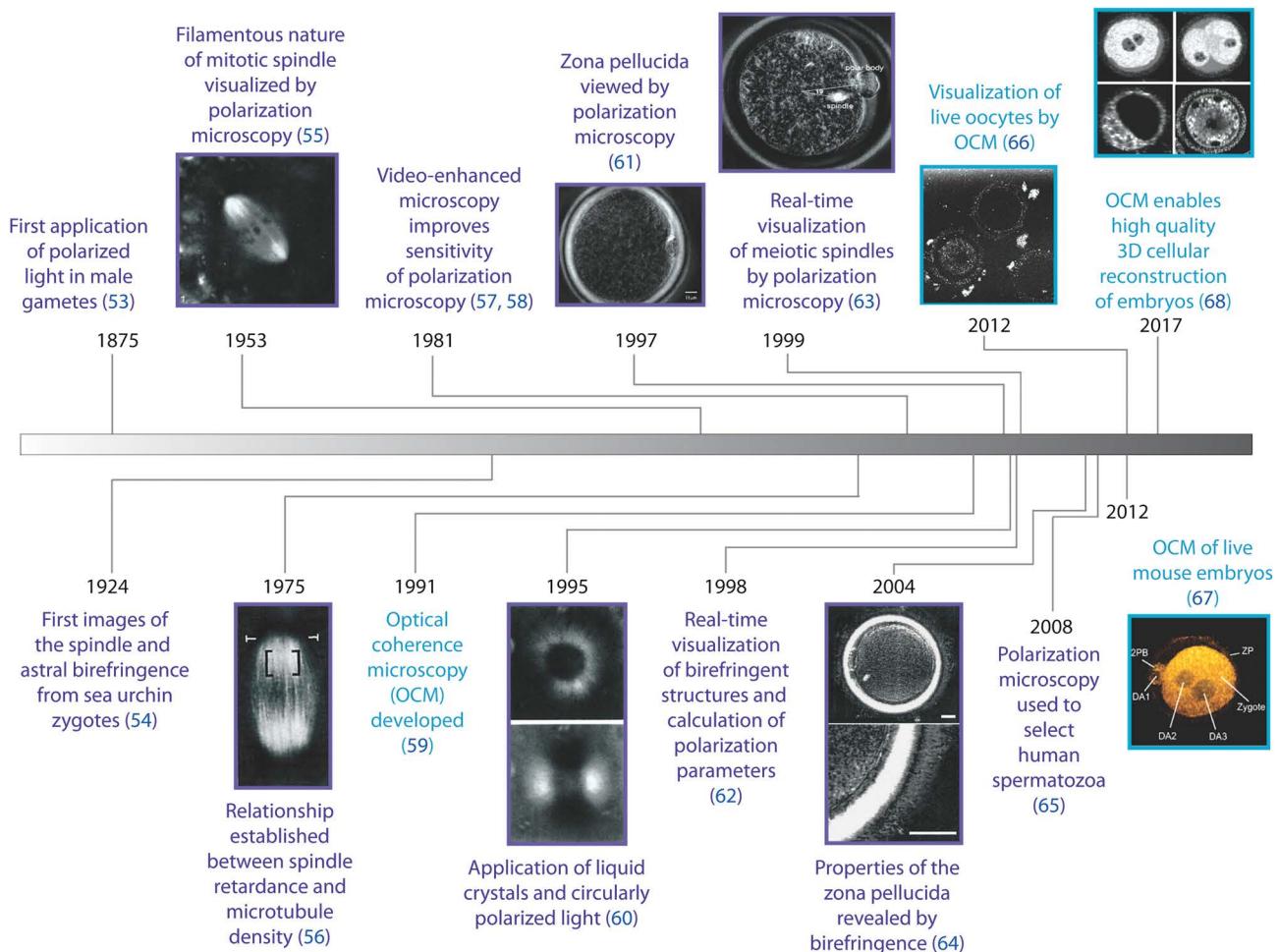


FIGURE 31.10 Timeline of alternative approaches for non-invasive label-free live imaging. (Images reproduced with permission from [55, 56, 60, 61, 63, 64, 66] (© The Optical Society), and [67, 68]).

Characterization of the architecture of the zona pellucida of human oocytes with polarization microscopy revealed an association between the birefringence of the inner layer and the developmental potential of an oocyte [76]. Subsequent studies confirmed an association between the birefringence of the zona pellucida and blastocyst formation, implantation, and pregnancy rates [77–79], although the underlying mechanisms remain to be determined.

Optical coherence microscopy (OCM)

OCM is a relatively new non-invasive technology for label-free imaging which generates 3D reconstructions based on intrinsic contrasting of back-scattered coherent light [59]. OCM has proven to be a very useful technique for embryonic developmental imaging particularly due to non-invasive depth-resolved imaging, rapid acquisition speed, and high spatial resolution. It has been named the “optical biopsy” due to the similarities between cross-sectional OCM images of different embryos and their histological sections [80]. Moreover, OCM can distinguish between normal and abnormal embryonic morphology [81].

In mammalian embryos, OCM can provide images of critical intracellular organelles like nuclei and nucleoli, metaphase spindles, networks of endoplasmic reticulum and mitochondria and, most importantly, may be used to monitor and quantitatively analyse their dynamic behaviour and evolution over time (Figure 31.10) [68]. Although OCM has yet to be translated into the IVF clinic, its capacity for high-resolution label-free imaging and its rapid uptake in other fields of medicine such as ophthalmology, suggest this technology may become an invaluable tool to both further our understanding of human embryo development and improve assisted reproductive outcomes.

Artificial intelligence for embryo selection

In recent years, there has been an increasing focus on using artificial intelligence (AI) to identify the best embryos for transfer. The advantage of this approach is that it can be non-invasive, requiring only a static image of the embryo and removes the potential variability introduced by different embryologists. Generally, this type of analysis requires computational segmentation of various features within an image of an embryo and the application of a model based on machine learning to predict the developmental potential. The models are trained by iterative learning from thousands of example images of embryos with known outcomes and do not depend on the specification of features by humans [82, 83].

Machine learning approaches can rank embryos based on quality at least as well as embryologists and can even outperform highly experienced embryologists in selecting between good quality blastocysts for implantation potential, biopsy, or cryopreservation [84–86]. Models are now being developed with the aim of improving each step of the ART process from fertilization to implantation and clinical pregnancy (reviewed in [87]). Whilst AI has the potential to improve ART outcomes, there are important limitations which must be considered. Machine learning is very sensitive to data quality and most AI systems do not adapt well to data acquired on different imaging systems or changes in imaging parameters. Indeed, embryo scores have been shown to be affected by the imaging magnification and the focal plane of the image capture [88]. This poses problems when attempting to use AI with data acquired on various systems at different clinics. Very few clinics have the same expensive imaging systems used to

produce the training datasets. One potential solution is to retrain the algorithm using lower quality data acquired on in-house systems which may include inexpensive portable cameras or even smartphone-based systems [89]. In addition, training data sets often contain images of embryos that failed to implant. However, it is not possible to know if this failure is due to a problem with the embryo or adverse maternal factors, making the data less reliable.

Given the critical role of the maternal uterine environment in the establishment of a viable pregnancy, AI prediction of implantation will always be limited. Nevertheless, AI analysis of embryo images can improve consistency in embryo selection and while it is still a long way from replacing embryologists, AI is a useful tool to enhance the performance of trained embryologists [90].

Conclusion

Since the first staging of human embryos by Franklin P. Mall in 1914, static images have provided a wealth of information about early human development. Recent advances in *in vitro* culture and implantation of human embryos are providing an unprecedented view of the cellular and molecular events directing early embryogenesis. Coupling these approaches with new imaging technologies and advances in computational image analysis will yield new insights that could improve human embryo culture and selection for assisted reproduction.

References

1. Hertig AT, Rock J, Adams EC. A description of 34 human ova within the first 17 days of development. Am J Anat. 1956;98(3):435–93.
2. Weimar CH, Post Uiterweer ED, Teklenburg G, Heijnen CJ, Macklon NS. In-vitro model systems for the study of human embryo-endometrium interactions. Reprod Biomed Online. 2013; 27(5):461–76.
3. Shahbazi MN, Jedrusik A, Vuoristo S, et al. Self-organization of the human embryo in the absence of maternal tissues. Nat Cell Biol. 2016;18(6):700–8.
4. Deglincerti A, Croft GF, Pietila LN, et al. Self-organization of the in vitro attached human embryo. Nature. 2016;533(7602):251–4.
5. Xiang L, Yin Y, Zheng Y, et al. A developmental landscape of 3D-cultured human pre-gastrulation embryos. Nature. 2020; 577(7791):537–42.
6. Scott LA, Smith S. The successful use of pronuclear embryo transfers the day following oocyte retrieval. Hum Reprod. 1998;13(4):1003–13.
7. Scott L, Alvero R, Leondires M, Miller B. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. Hum Reprod. 2000;15(11):2394–403.
8. Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. Hum Reprod. 1999;14(5):1318–23.
9. Tesarik J, Junca AM, Hazout A, et al. Embryos with high implantation potential after intracytoplasmic sperm injection can be recognized by a simple, non-invasive examination of pronuclear morphology. Hum Reprod. 2000;15(6):1396–9.
10. James AN, Hennessy S, Reggio B, et al. The limited importance of pronuclear scoring of human zygotes. Hum Reprod. 2006;21(6):1599–604.
11. Nicoli A, Valli B, Di Girolamo R, et al. Limited importance of pre-embryo pronuclear morphology (zygote score) in assisted reproduction outcome in the absence of embryo cryopreservation. Fertil Steril. 2007;88(4 Suppl):1167–73.
12. Weitzman VN, Schnee-Riesz J, Benadiva C, et al. Predictive value of embryo grading for embryos with known outcomes. Fertil Steril. 2010;93(2):658–62.

13. Berger DS, Zapantis A, Merhi Z, et al. Embryo quality but not pro-nuclear score is associated with clinical pregnancy following IVF. *J Assist Reprod Genet.* 2014;31(3):279–83.
14. Vanneste E, Voet T, Le Caignec C, et al. Chromosome instability is common in human cleavage-stage embryos. *Nat Med.* 2009;15(5):577–83.
15. Kort DH, Chia G, Treff NR, et al. Human embryos commonly form abnormal nuclei during development: A mechanism of DNA damage, embryonic aneuploidy, and developmental arrest. *Hum Reprod.* 2016;31(2):312–23.
16. Jacobs K, Van de Velde H, De Paepe C, Sermon K, Spits C. Mitotic spindle disruption in human preimplantation embryos activates the spindle assembly checkpoint but not apoptosis until day 5 of development. *Mol Hum Reprod.* 2017;23(5):321–29.
17. Cauffman G, Verheyen G, Tournaye H, Van de Velde H. Developmental capacity and pregnancy rate of tetrahedral- versus non-tetrahedral-shaped 4-cell stage human embryos. *J Assist Reprod Genet.* 2014;31(4):427–34.
18. Antczak M, Van Blerkom J. Oocyte influences on early development: The regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Mol Hum Reprod.* 1997;3(12):1067–86.
19. Desai N, Gill P. Blastomere cleavage plane orientation and the tetrahedral formation are associated with increased probability of a good-quality blastocyst for cryopreservation or transfer: A time-lapse study. *Fertil Steril.* 2019;111(6):1159–68.e1.
20. Nikas G, Ao A, Winston RM, Handyside AH. Compaction and surface polarity in the human embryo in vitro. *Biol Reprod.* 1996;55(1):32–7.
21. Iwata K, Yumoto K, Sugishima M, et al. Analysis of compaction initiation in human embryos by using time-lapse cinematography. *J Assist Reprod Genet.* 2014;31(4):421–6.
22. Mizobe Y, Ezono Y, Tokunaga M, et al. Selection of human blastocysts with a high implantation potential based on timely compaction. *J Assist Reprod Genet.* 2017;34(8):991–7.
23. Alikani M. Epithelial cadherin distribution in abnormal human pre-implantation embryos. *Hum Reprod.* 2005;20(12):3369–75.
24. Zhu M, Shahbazi M, Martin A, et al. Human embryo polarization requires PLC signaling to mediate trophectoderm specification. *Elife.* 2021;10:e65068.
25. Gerri C, McCarthy A, Alanis-Lobato G, et al. Initiation of a conserved trophectoderm program in human, cow and mouse embryos. *Nature.* 2020;587(7834):443–7.
26. Koot YE, Teklenburg G, Salker MS, Brosens JJ, Macklon NS. Molecular aspects of implantation failure. *Biochim Biophys Acta.* 2012;1822(12):1943–50.
27. Fransasiak JM, Alecsandru D, Forman EJ, et al. A review of the pathophysiology of recurrent implantation failure. *Fertil Steril.* 2021;116(6):1436–48.
28. Aberkane A, Essahib W, Spits C, et al. Expression of adhesion and extracellular matrix genes in human blastocysts upon attachment in a 2D co-culture system. *Mol Hum Reprod.* 2018;24(7):375–387.
29. Lindenberg S. Ultrastructure in human implantation: Transmission and scanning electron microscopy. *Baillieres Clin Obstet Gynaecol.* 1991;5(1):1–14.
30. Grewal S, Carver JG, Ridley AJ, Mardon HJ. Implantation of the human embryo requires Rac1-dependent endometrial stromal cell migration. *Proc Natl Acad Sci U S A.* 2008;105(42):16189–94.
31. Kang YJ, Forbes K, Carver J, Aplin JD. The role of the osteopontin-integrin alphavbeta3 interaction at implantation: Functional analysis using three different in vitro models. *Hum Reprod.* 2014;29(4):739–49.
32. Bontekoe S, Mantikou E, van Wely M, et al. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Database Syst Rev.* 2012;(7):CD008950. doi: 10.1002/14651858.CD008950.pub2.
33. Meintjes M, Chantilis SJ, Douglas JD, et al. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. *Hum Reprod.* 2009;24(2):300–7.
34. Mole MA, Weberling A, Fassler R, et al. Integrin beta1 coordinates survival and morphogenesis of the embryonic lineage upon implantation and pluripotency transition. *Cell Rep.* 2021;34(10):108834.
35. Shahbazi MN, Scialdone A, Skorupska N, et al. Pluripotent state transitions coordinate morphogenesis in mouse and human embryos. *Nature.* 2017;552(7684):239–43.
36. Mole MA, Coorens THH, Shahbazi MN, et al. A single cell characterisation of human embryogenesis identifies pluripotency transitions and putative anterior hypoblast centre. *Nat Commun.* 2021;12(1):3679.
37. Schumacher A, Zenclussen AC. Human chorionic gonadotropin-mediated immune responses that facilitate embryo implantation and placentation. *Front Immunol.* 2019;10:2896.
38. Poikkeus P, Hiilesmaa V, Tiitinen A. Serum HCG 12 days after embryo transfer in predicting pregnancy outcome. *Hum Reprod.* 2002;17(7):1901–5.
39. Rull K, Laan M. Expression of beta-subunit of HCG genes during normal and failed pregnancy. *Hum Reprod.* 2005;20(12):3360–8.
40. Bentin-Ley U, Horn T, Sjogren A, et al. Ultrastructure of human blastocyst-endometrial interactions in vitro. *J Reprod Fertil.* 2000;120(2):337–50.
41. Shahbazi MN, Wang T, Tao X, et al. Developmental potential of aneuploid human embryos cultured beyond implantation. *Nat Commun.* 2020;11(1):3987.
42. Ruane PT, Garner T, Parsons L, et al. Trophectoderm differentiation to invasive syncytiotrophoblast is promoted by endometrial epithelial cells during human embryo implantation. *Hum Reprod.* 2022;37(4):777–92.
43. Weberling A, Zernicka-Goetz M. Trophectoderm mechanics direct epiblast shape upon embryo implantation. *Cell Rep.* 2021;34(3):108655.
44. Enders AC. Trophoblast differentiation during the transition from trophoblastic plate to lacunar stage of implantation in the rhesus monkey and human. *Am J Anat.* 1989;186(1):85–98.
45. National Research Council (US) and Institute of Medicine (US) Human Embryonic Stem Cell Research Advisory Committee. Final Report of the National Academies' Human Embryonic Stem Cell Research Advisory Committee and 2010 Amendments to the National Academies' Guidelines for Human Embryonic Stem Cell Research. Washington (DC): National Academies Press (US), 2010.
46. Vilska S, Tiitinen A, Hyden-Granskog C, Hovatta O. Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. *Hum Reprod.* 1999;14(9):2392–5.
47. Neubourg DD, Gerris J. Single embryo transfer – state of the art. *Reprod Biomed Online.* 2003;7(6):615–22.
48. Grady R, Alavi N, Vale R, Khandwala M, McDonald SD. Elective single embryo transfer and perinatal outcomes: A systematic review and meta-analysis. *Fertil Steril.* 2012;97(2):324–31.
49. Edwards RG, Fishel SB, Cohen J, et al. Factors influencing the success of in vitro fertilization for alleviating human infertility. *J In Vitro Fert Embryo Transf.* 1984;1(1):3–23.
50. M Alpha Scientists in Reproductive, ESGO Embryology. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Hum Reprod.* 2011;26(6):1270–83.
51. Basile N, Elkhatib I, Meseguer M. A strength, weaknesses, opportunities and threats analysis on time lapse. *Curr Opin Obstet Gynecol.* 2019;31(3):148–55.
52. Armstrong S, Bhide P, Jordan V, et al. Time-lapse systems for embryo incubation and assessment in assisted reproduction. *Cochrane Database Syst Rev.* 2019;5:CD011320.
53. Engelmann TW. Contractilität und Doppelbrechung. Archiv für die gesamte Physiologie des Menschen und der Tiere. 1875; 11(1):432–64.

54. Schmidt WJ. Die bausteine des tierkörpers in polarisiertem lichte. Bonn, Germany: Friedrich Cohen, 1924.
55. Inoue S. Polarization optical studies of the mitotic spindle. I. The demonstration of spindle fibers in living cells. *Chromosoma*. 1953;5(5):487–500.
56. Sato H, Ellis GW, Inoue S. Microtubular origin of mitotic spindle form birefringence. Demonstration of the applicability of Wiener's equation. *J Cell Biol*. 1975;67(3):501–17.
57. Allen RD, Travis JL, Allen NS, Yilmaz H. Video-enhanced contrast polarization (AVEC-POL) microscopy: A new method applied to the detection of birefringence in the motile reticulopodial network of *Allogromia laticollaris*. *Cell Motil*. 1981;1(3):275–89.
58. Inoue S. Video image processing greatly enhances contrast, quality, and speed in polarization-based microscopy. *J Cell Biol*. 1981;89(2):346–56.
59. Huang D, Swanson EA, Lin CP, et al. Optical coherence tomography. *Science*. 1991;254(5035):1178–81.
60. Oldenbourg R, Mei G. New polarized light microscope with precision universal compensator. *J Microsc*. 1995;180(Pt 2):140–7.
61. Keefe D, Tran P, Pellegrini C, Oldenbourg R. Polarized light microscopy and digital image processing identify a multilaminar structure of the hamster zona pellucida. *Hum Reprod*. 1997;12(6):1250–2.
62. Oldenbourg R, Salmon ED, Tran PT. Birefringence of single and bundled microtubules. *Biophys J*. 1998;74(1):645–54.
63. Silva CP, Kommineni K, Oldenbourg R, Keefe DL. The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. *Fertil Steril*. 1999;71(4):719–21.
64. Pelletier C, Keefe DL, Trimarchi JR. Noninvasive polarized light microscopy quantitatively distinguishes the multilaminar structure of the zona pellucida of living human eggs and embryos. *Fertil Steril*. 2004;81(Suppl 1):850–6.
65. Gianaroli L, Magli MC, Collodel G, et al. Sperm head's birefringence: A new criterion for sperm selection. *Fertil Steril*. 2008;90(1):104–12.
66. Xiao J, Wang B, Lu G, Zhu Z, Huang Y. Imaging of oocyte development using ultrahigh-resolution full-field optical coherence tomography. *Appl Opt*. 2012;51(16):3650–4.
67. Zheng JG, Lu D, Chen T, et al. Label-free subcellular 3D live imaging of preimplantation mouse embryos with full-field optical coherence tomography. *J Biomed Opt*. 2012;17(7):070503.
68. Karnowski K, Ajduk A, Wieloch B, et al. Optical coherence microscopy as a novel, non-invasive method for the 4D live imaging of early mammalian embryos. *Sci Rep*. 2017;7(1):4165.
69. Kuhn JR, Wu Z, Poenie M. Modulated polarization microscopy: A promising new approach to visualizing cytoskeletal dynamics in living cells. *Biophys J*. 2001;80(2):972–85.
70. Rienzi L, Martinez F, Ubaldi F, et al. Polscope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. *Hum Reprod*. 2004;19(3):655–9.
71. De Santis L, Cino I, Rabellotti E, et al. Polar body morphology and spindle imaging as predictors of oocyte quality. *Reprod Biomed Online*. 2005;11(1):36–42.
72. Montag M, Schimming T, van der Ven H. Spindle imaging in human oocytes: The impact of the meiotic cell cycle. *Reprod Biomed Online*. 2006;12(4):442–6.
73. Shen Y, Betzendahl I, Tinneberg HR, Eichenlaub-Ritter U. Enhanced polarizing microscopy as a new tool in aneuploidy research in oocytes. *Mutat Res*. 2008;651(1-2):131–40.
74. Omidi M, Faramarzi A, Agharrahimi A, Khalili MA. Noninvasive imaging systems for gametes and embryo selection in IVF programs: A review. *J Microsc*. 2017;267(3):253–64.
75. Petersen CG, Oliveira JB, Mauri AL, et al. Relationship between visualization of meiotic spindle in human oocytes and ICSI outcomes: A meta-analysis. *Reprod Biomed Online*. 2009;18(2):235–43.
76. Shen Y, Stalf T, Mehnert C, Eichenlaub-Ritter U, Tinneberg HR. High magnitude of light retardation by the zona pellucida is associated with conception cycles. *Hum Reprod*. 2005;20(6):1596–606.
77. Raju GAR, Prakash GJ, Krishna KM, Madan K. Meiotic spindle and zona pellucida characteristics as predictors of embryonic development: A preliminary study using PolScope imaging. *Reprod Biomed Online*. 2007;14(2):166–74.
78. Montag M, Schimming T, Koster M, et al. Oocyte zona birefringence intensity is associated with embryonic implantation potential in ICSI cycles. *Reprod Biomed Online*. 2008;16(2):239–44.
79. Ebner T, Balaban B, Moser M, et al. Automatic user-independent zona pellucida imaging at the oocyte stage allows for the prediction of preimplantation development. *Fertil Steril*. 2010;94(3):913–20.
80. Boppert SA, Brezinski ME, Bouma BE, Tearney GJ, Fujimoto JG. Investigation of developing embryonic morphology using optical coherence tomography. *Dev Biol*. 1996;177(1):54–63.
81. Boppert SA, Bouma BE, Brezinski ME, Tearney GJ, Fujimoto JG. Imaging developing neural morphology using optical coherence tomography. *J Neurosci Methods*. 1996;70(1):65–72.
82. Khosravi P, Kazemi E, Zhan Q, et al. Deep learning enables robust assessment and selection of human blastocysts after in vitro fertilization. *NPJ Digit Med*. 2019;2:21.
83. Dimitriadis I, Bormann C, Thirumalaraju P, et al. Artificial intelligence-enabled system for embryo classification and selection based on image analysis. *Fertil Steril*. 2019;111(4):e21.
84. Bormann CL, Kanakasabapathy MK, Thirumalaraju P, et al. Performance of a deep learning based neural network in the selection of human blastocysts for implantation. *Elife*. 2020;9:e55301.
85. Bormann CL, Thirumalaraju P, Kanakasabapathy MK, et al. Consistency and objectivity of automated embryo assessments using deep neural networks. *Fertil Steril*. 2020;113(4):781–7.e1.
86. Chavez-Badiola A, Flores-Saiffe-Farias A, Mendizabal-Ruiz G, Drakeley AJ, Cohen J. Embryo ranking intelligent classification algorithm (ERICA): Artificial intelligence clinical assistant predicting embryo ploidy and implantation. *Reprod Biomed Online*. 2020;41(4):585–93.
87. Dimitriadis I, Zaninovic N, Badiola AC, Bormann CL. Artificial intelligence in the embryology laboratory: A review. *Reprod Biomed Online*. 2022;44(3):435–48.
88. Loewke K, Cho JH, Brumar CD, et al. Characterization of an artificial intelligence model for ranking static images of blastocyst stage embryos. *Fertil Steril*. 2022;117(3):528–35.
89. Kanakasabapathy MK, Thirumalaraju P, Bormann CL, et al. Development and evaluation of inexpensive automated deep learning-based imaging systems for embryology. *Lab Chip*. 2019;19(24):4139–45.
90. Fitz VW, Kanakasabapathy MK, Thirumalaraju P, et al. Should there be an "AI" in TEAM? Embryologists selection of high implantation potential embryos improves with the aid of an artificial intelligence algorithm. *J Assist Reprod Genet*. 2021;38(10):2663–70.



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Textbook of Assisted Reproductive Techniques

Sixth Edition

Volume 2: Clinical Perspectives



Edited by
DAVID K. GARDNER
ARIEL WEISSMAN
COLIN M. HOWLES
ZEEV SHOHAM

Textbook of Assisted Reproductive Techniques

Established as the definitive reference for the IVF clinic, this Sixth Edition has been extensively revised, with the addition of several important new contributions on clinical topics, including the use of digitalization and precision medicine in the IVF clinic, the environment and reproduction, the use of gonadotropin-releasing hormone agonists and the efficiency of IVF, controlled ovarian stimulation for freeze-all cycles, immunology in ART, home monitoring of ART cycles, luteal-phase support in ART, the POSEIDON stratification of "low prognosis" patients in ART, controlled ovarian stimulation for low-responder patients, adjuvants for poor responders, innovative therapies in diminished ovarian reserve and primary ovarian insufficiency patients, and fertility options for transgender and nonbinary individuals. As previously, methods, protocols, and techniques of choice are presented by IVF pioneers and eminent international experts.

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PREFACE

The first edition of the *Textbook of Assisted Reproductive Techniques* was published in 2001. As the textbook now enters its sixth edition, some 45 years since the birth of Louise Brown, the world's first test tube baby in the United Kingdom, it is remarkable to reflect upon the changes in assisted human conception that have been documented in each successive edition of the textbook.

Over the past 20 years, we have witnessed the widespread implementation of single blastocyst transfer, and the ability to undertake trophectoderm biopsy and genetic analysis now using next-generation sequencing to accurately determine chromosomal copy number, and to provide precise genetic diagnosis for patients as needed. This shift in practice of transferring only one high-quality embryo has brought us closer to the mantra of "one embryo, one baby." Cryopreservation, historically performed using slow-rate controlled freezing, has now been superseded by vitrification for both oocytes and embryos, with oocyte cryopreservation becoming a realistic treatment for fertility preservation, especially for oncology patients and younger women wishing to preserve their fertility. Improvements in laboratory culture techniques and incubation devices, including time-lapse imaging, have also contributed to the adoption of single-embryo transfers without reducing the chance of a live birth. Excitingly, more technologies are now available for sperm assessment, and the knowledge underpinning *in vitro* maturation has facilitated the development of potential new approaches for IVF.

As for ovarian stimulation protocols, there has been, over the past 20 years of this textbook series, a major shift in practice. The clinical acceptance of the GnRH antagonist protocol, first registered in 1999, took more than 10 years to be widely adopted. With the possibility of using a GnRH agonist to trigger follicular

maturity, the protocol has become the preferred choice, facilitating the concept of an "OHSS-free clinic." A plethora of new pharmaceutical FSH agents have been introduced into practice that have resulted in increased patient convenience and drug delivery precision (due to use of pen devices) rather than increased live birth rates. This is a further reflection of the complexity of the overall IVF treatment process—in particular, the pivotal role that the embryology laboratory continues to play in improving cycle success.

Sadly, however, over the duration of this textbook's life span, we have lost several authors—all dear friends and colleagues—whom we miss and to whom we are grateful for their enormous contributions to our field during their lifetimes:

- Marinko Biljan, Quebec
- Isaac Blickstein, Rehovot
- Jean Cohen, Paris
- Howard W Jones Jr, Norfolk
- Michelle Lane, Adelaide
- Ragaa Mansour, Egypt
- Queenie V Neri, New York
- Lynette Scott, Boston
- Carl Wood, Melbourne
- Yury Velinsky, Chicago

Finally, we lost one of the pioneering fathers of this field, Bob Edwards, a giant in our field on whose shoulders we have all been fortunate to stand.

**David K. Gardner, Ariel Weissman,
Colin M. Howles, and Zeev Shoham**



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QUALITY MANAGEMENT IN REPRODUCTIVE MEDICINE

Michael Alper

Introduction

Quality management systems (QMSs) have become integral management tools in many *in vitro* fertilization (IVF) centres around the world. The European Union (EU) Tissue Directive, issued in 2004, clearly demands a QMS for any institution handling human gametes/embryos. The primary concerns of any healthcare system will continue to be clinical outcomes. However, if we regard medical facilities as businesses providing a particular service to patients and referring doctors, then other parameters beyond clinical outcomes become important. Governmental agencies and insurance companies will continue to place increasing pressure on documenting that they provide services in a particular fashion. This will mean that strict procedures for documentation of results will be needed and, furthermore, practices could be penalized if they do not perform adequately. Governmental agencies control some practices through regulations (e.g. certain infection disease protocols). However, beyond these rules, many medical organizations currently develop their own internal standards. These standards are often only informally documented and most of the time are fragmentary. These standards affect and direct the internal workings of the organization and the interactions of various areas within the company. They may also affect the interactions of the company with external partners. For example, if every institution were to use their own internally developed methodology for documenting and handling different procedures, then it would be very difficult to compare and contrast different systems. A customizable single system to follow all the internal workings of the organization is the goal of the QMSs, such as that of the International Organization for Standardization (ISO; see the following section). It is important to recognize that an organization has many “customers.” Often clinicians feel uncomfortable referring to patients as customers, but of course patients are our key customers. But other “customers” exist and include referring doctors, insurance companies, regulatory bodies, and students, among others. Another set of key customers consists of our employees—our “internal customers.”

The individual elements of a QMS are developed to different degrees, but always according to the tasks and the orientation of the particular institution. They exist in a varied yet well-defined relationship with one another. All of these elements and their interconnections as a whole enable a clinic or private practice to reach the expected and agreed results with the customer on a timely basis, and with an appropriate use of resources. The sum of directive elements and elements that transcend or relate to the process is called the “QMS” of a clinic or a private practice. Compared with other medical specialties, reproductive medicine has led the way (in Europe) with the introduction of QMSs over the past several years. In this chapter, different QMSs are described, the instruments of these systems are discussed, and

the question of how QMSs contribute to success in reproductive medicine is addressed.

Different QMSs

Several industry-specific QMSs have been developed worldwide. In 1964, Good Production Practice (a World Health Organization [WHO] directive) was developed for the pharmaceutical and food industries. Good Laboratory Practice (an Organization for Economic Cooperation and Development [OECD] directive) followed in 1978, as did the Hazard Analysis of Critical Control Points (a National Advisory Committee on Microbiological Criteria for Foods directive) in 1992. The EU, with its “Global Concept” (1985), strongly promoted the development of QMSs and expanded them to production and services.

ISO 9001 standards

The systems that followed—i.e. the manuals of the ISO (the ISO 9000 series)—became the most widespread worldwide standard. In the 1980s, the ISO created regulations for QMSs with the standard series 9001 through 9004 developed for the production of goods and services. These manuals described the basic elements of the QMS in a relatively abstract manner. Medical institutions were required to adapt these standards to the medical field, which required some interpretation and modification. The introduction of ISO 9000 states: “The demands of the organizations differ from each other; during the creation of quality management systems and putting them into practice, the special goals of the organization, its products and procedures and specific methods of acting must be taken into consideration unconditionally.” This means that, for medical applications, the standards state which elements should be considered in the QMS, but the manner in which these elements should be realized in the specific medical organization must be defined individually. Furthermore, specific interpretation of the ISO for IVF centres is limited. The ISO standards have now been adapted to medicine, which is fortunate since there is no QMS specifically designed for hospitals or medical practices. ISO 9001 through 9003 contain the elements that are important for a quality system ([Table 32.1](#)). The criteria according to which QMSs are applied vary with the type of enterprise. For example, the 9001 standard applies to manufacturing and complicated service companies, including hospitals and medical practices. On the other hand, the 9002 standard is more suitable for rehabilitation and foster-care institutions [\[1\]](#). The application of a certified QMS for hospitals can be performed on the basis of ISO 9001 or ISO 9004 [\[2\]](#). More recent publications describing the application of ISO to IVF centres are now available (see the textbook by Carson et al.). As mentioned earlier, IVF units occupy a special place within clinical medicine. This is a highly specialized area that involves the interaction of staff in various areas, including the laboratory, ultrasound, administration, physicians, and

TABLE 32.1 Elements/Criteria of the International Organization for Standardization (ISO) Standard

Number	Quality Element According to ISO 9000 ff.
1	Responsibility
2	Quality management system
3	Contract control
4	Design management
5	Document and data management
6	Measures
7	Management of products provided for customers
8	Designating and retrospective observation
9	Process management
10	Revision
11	Control of the revision resources
12	Evidence of revisions
13	Defective product management
14	Corrections and preventive measures
15	Handling, storage, packaging, conservation, and distribution
16	Quality report management
17	Internal quality audits
18	Training
19	Maintenance
20	Statistical methods

nurses. Treatment can only be successful when a structured interaction exists between the clinical and laboratory departments. ISO 9001 [3] is very much focused on a process approach and is directed at the outcome of the process (i.e. that the products or services meet the previously determined requirements). Since this does not necessarily ensure that a laboratory will be successful or pregnancy rates will be as good as possible, or that it will achieve the highest level of care for the patients that it serves, assisted reproduction technology (ART) laboratories may also want to consider additional requirements, including standards concerning qualifications and competence. Relevant standards are provided by the ISO/IEC 17025:1999 [4] (IEC being the International Electrotechnical Commission). This standard, entitled "General Requirements for the Competence of Testing and Calibration Laboratories," replaces both the ISO/IEC Guide 25 [5] and the European standard EN 45001 [6]. Compliance with the ISO 17025 standard can lead to accreditation (defined as "a procedure by which an authoritative body gives formal recognition that a body or person is competent to carry out specific tasks") which exceeds certification (defined as "a procedure by which a third party gives written assurance that a product, process or service conforms to specific requirements"). ART laboratories may want to consider ISO 17025 accreditation. However, one should realize that both ISO/IEC Guide 25 and EN 45001 are focused more on the technical aspects of competence, and do not cover all areas within clinical laboratories. It has already been stated that although the ISO standards are the most widely accepted standards in the world, there is no appropriate international standard for laboratories in the healthcare sector. To fulfil this need, several professional associations and laboratory organizations have also framed and published standards and guidelines, most of which are confined to a specific clinical laboratory discipline. Some specific and

relevant examples of guidelines for ART laboratories that are commonly available are [7–10]:

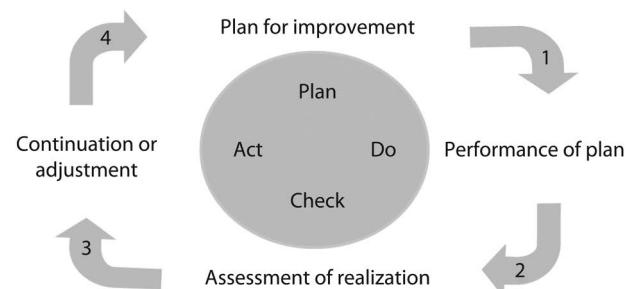
1. Revised Guidelines for Human Embryology and Andrology Laboratories, the American Fertility Society, 2008
2. Revised Guidelines for Good Practice in IVF Laboratories, the European Society of Human Reproduction and Embryology (ESHRE), 2008
3. Reproductive Laboratory Accreditation Standards, College of American Pathology, 2013
4. Accreditation Standards and Guidelines for IVF Laboratories, the Association of Clinical Embryologists, 2000

The aforementioned guidelines and standards describe the specific requirements for reproductive laboratories, and include various aspects of the implementation of a QMS. These well-defined standards describe the minimum conditions that should be met by laboratories and clinics. Recently, the EU Tissue Directive [11] has been released, which demands a QMS for every medical institution that deals with human gametes or embryos.

Total quality management and the excellence model of the EFQM

There is a wide range of QM models and strategies based on continuous improvement. Two of the best-documented models/strategies are Total Quality Management (TQM) and the Excellence Model of the European Foundation for Quality Management (EFQM). TQM is an all-encompassing concept that integrates quality control, assurance, and improvement. It is more of a philosophy than a model. Deming developed the basics of this concept after World War II. Both the TQM and the EFQM models incorporate the objective of continuously striving to improve every aspect of a service, and require continuous scrutiny of all components of the QMS of an organization. Measurement and feedback are crucial elements in QM. This can be illustrated by the so-called Deming cycle (the "Plan–Do–Check–Act" cycle) (Figure 32.1). Important elements of a TQM program are:

1. Appropriately educated and trained personnel with training records
2. Complete listing of all technical procedures performed
3. Housekeeping procedures: cleaning and decontamination procedures
4. Correct operation, calibration, and maintenance of all instruments with manuals and logbook records
5. Proper procedure policy and safety manuals
6. Consistent and proper execution of appropriate techniques and methods

**FIGURE 32.1** Total quality management: The Deming cycle.

7. Proper documentation, record-keeping, and reporting of results
8. Thorough description of specimen collection and handling, including verification procedures for patient identification and chain of custody
9. Safety procedures, including appropriate storage of materials
10. Infection control measures
11. Documentation of suppliers and sources of chemicals and supplies, with dates of receipt/expiry
12. System for appraisal of test performance correction of deficiencies and implementation of advances and improvements
13. Quality materials, tested with bioassays when appropriate
14. Quality assurance programs

Quality policy

One of the first steps for the implementation of a QMS in a medical institution is to clearly define the quality policy. Quality policies are a group of principles that establish the workings of the institution. Although successful treatment of an existing disease or reduction of discomfort is certainly the highest priority for most medical institutions, it might be an important goal to achieve this in the most efficient manner possible. This means that structure is needed to ensure that diagnostic and therapeutic procedures are performed using the most appropriate financial, organizational, or time resources available, while still striving for a high quality of treatment. After all, optimum quality is achieved by the “right” balance between cost and quality. The quality policy of a medical institution cannot be defined by a single person (e.g. the owner or medical director), but should be developed as a consensus between management and employees. Only in this way will personnel identify with the quality policy of the institution. A quality policy should be formulated in an active manner, and the formulation should also be short and simple so that every employee can repeat the quality policy at any time. The most important aspects of the quality policy should be posted in suitable and accessible areas of the institution for employees, patients, and visitors in order to strengthen the employees’ knowledge of common goals, improve their identification with their own areas of competence, and communicate these principles to others. It is important to state that quality policies should be reviewed periodically to make sure that the principles are still valid and that management and employees still agree with them. As an organization’s perspectives and goals change, the quality policy needs to be modified accordingly. As an example, Boston IVF’s quality policy is “CARE,” standing for Compassionate, Advanced, Responsive, and Experienced.

Management’s responsibility

In spite of the fact that the responsibility of management (or the governing structure) can be defined differently in various medical institutions, according to ISO standards, certain generally valid aspects can be defined. The hierarchy of the institution has to be defined and outlined clearly. Although larger institutions commonly have clear charts of who reports to whom, the structure might be more challenging to delineate in private centres with multiple partners in equal positions. In such cases, an agreement that describes the division of responsibilities for

particular fields among the physicians must be in place. Several possibilities are available; for example, one of the partners could be in charge of research and another could be in a business role. However, for many privately held practices, a model may exist for dividing these tasks on a rotational basis. It is here that clear descriptions of authority for all positions within the organization are required and must be known to everyone, both internally and externally. The more complex the hierarchic structures within a medical institution, the more precisely these structures must be defined for the system to work effectively and robustly at all times and under all (extraordinary) conditions. The “decision-maker” of the head of the organization must be available at any time, even if he or she is physically absent. Therefore, it must be absolutely clear to everyone within the organization who has the competence and authority to make decisions. If the “decision-maker” is not available, then someone in the organization should be identified to make decisions in his/her absence. It is also important for customers outside of the company to be aware of who the decision-makers are for various tasks. There are various ways of making these structures as transparent as possible. One easy way is the development of an organizational chart ([Figure 32.2](#)). This organizational diagram can be placed in a suitable and accessible location, helping employees to understand everyone’s roles and responsibilities. Furthermore, making the organizational diagram available to everyone strengthens trust, cooperation, and professionalism within the company. It is also important for communication with patients, interested parties, or cooperating departments. The organizational diagram should be updated frequently. Management should strongly support the quality policies of the company and should take an active part in their development and implementation. It is important to lead by example.

Management of processes

Processes are all of the procedures that are necessary for the completion of tasks. For medical facilities, the most important processes are those of diagnostic and therapeutic procedures. In addition, many other processes are involved in the care of patients, such as the scheduling of patients for tests, communication, and anything else that may greatly affect a patient’s (customer’s) perspective. Sometimes poor communication can ruin a patient’s experience, despite the best diagnostic procedures within the organization. In fact, it is our observation that it is more likely that a patient will leave a medical facility because of an organizational problem such as a substandard secretarial or administrative problem than a medical deficiency. Even with properly working medical treatment, poor communication with colleagues can endanger or directly destroy the positive result of the treatment. When establishing a QMS, it is necessary to precisely define and describe all relevant processes and to structure them according to QM guidelines. These descriptions are often best realized by flow diagrams that can overlap in various places. These areas of contact between two flow diagrams are called boundaries, interferences, joints, or areas of juncture.

Documentation in a QMS

In addition to defining the processes that are relevant to the system, it is important for everything to be documented. The different levels of documentation are shown in [Figure 32.3](#). One of the most important documents in a QMS is the quality manual.

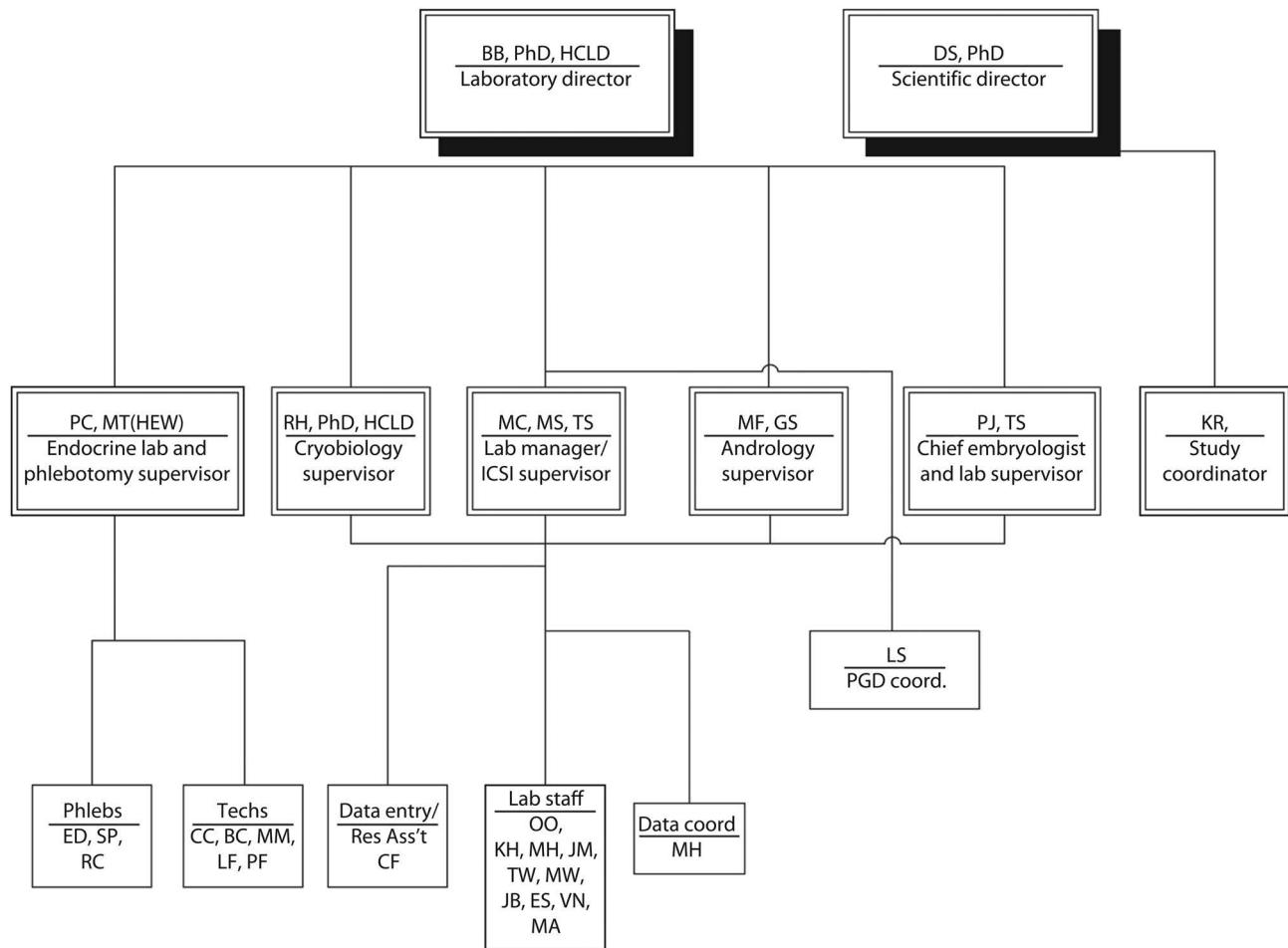


FIGURE 32.2 Example of an organizational chart.

The main purpose of the quality manual is to outline the structure of the documentation used in the quality system [12]. It should also include or refer to the standard operating procedures (SOPs). There should be clear definitions of management's areas of responsibility, including its responsibility for ensuring compliance with the international standards on which the system is built. A simple overview of the quality system requirements and

their position in the quality manual are shown in **Figure 32.4**. A good-quality manual should be precise and brief; it should be an easily navigable handbook for the entire quality system. The most important procedures are preferably included in the manual itself, but deeper descriptions should be referred to in the underlying documentation. An easy way to start building a system is to make up a table of contents for the quality manual and to decide

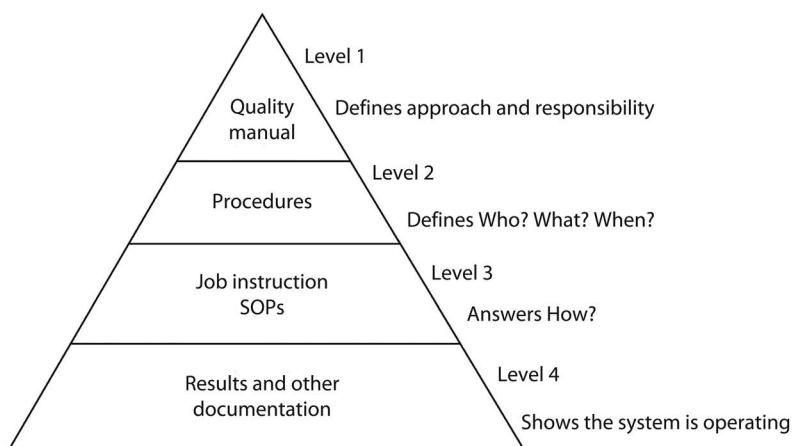


FIGURE 32.3 Levels of documentation. Abbreviation: SOP, standard operating procedure.

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Attachments:

- Boston IVF organizational chart
- Surgery Center of Waltham organizational chart
- Document Control Procedure
- Control of Quality Records Procedure
- Internal Audit Procedure
- Corrective Action Procedure
- Preventive Action Procedure
- Non-conforming Material Procedure

FIGURE 32.4 Example of an International Organization for Standardization quality manual.

which processes should be described in the manual and which should rather be described in the underlying documentation (e.g. SOPs). Whereas the quality manual contains more general information, the individual processes and procedures are described in a more detailed way in handbooks/job instructions or SOPs. These SOPs go through the processes step by step and describe the materials and methods used and the way the process is performed precisely. SOP manuals should be available to all personnel, and every single procedure in these manuals must be fully documented with signature, date, and regular review.

Document control

According to ISO 9001:2015, the clinic should establish and maintain procedures to control all documents that form part of its quality documentation. This includes both internally generated documentation, such as SOPs and protocol sheets, and externally generated documentation, such as law texts, standards, and instruction manuals for equipment. Document handling and control are important parts of the quality system and, if not designed properly, can become enormous burdens for a smooth-running system. Since it is something that touches every part of the system, it is important to sit down and think through how this system of paperwork is best handled in your clinic and to ensure that the system you choose covers the demands of the standards. The identification of the documents should be logical, and it is a good suggestion to use numbers as unique identifiers. The same identification number could then be used for the file name within

the computerized version. The issue number in parentheses or after a dash could follow this number. Pagination is important. If you choose not to use pagination, you must clearly mark where the document starts and ends. The dates of issue, together with information on who wrote the document and who approved it (with an authorized signature), are usually included in the document header. Questions that should have an answer in your document control system include:

1. Is *all* documentation in the laboratory or clinic covered by your document control system?
2. Who writes or changes the document?
3. Who approves and has the authority to issue documents?
4. Does the document have:
 - a. Unique identification?
 - b. Issue number and current revision status?
 - c. Date of latest issue?
 - d. Pagination?
5. Where can I find the document: physical location, level in the system, and on computer file?
6. Who ensures that only the latest issue of the document is present in the system, removes outdated issues, and files them?
7. Are amendments to documents clearly marked, initialled, and dated?
8. How are changes in a document implemented with the personnel?

Documentation of results

A very important level of documentation concerns “results.” This includes not only the results of treatment, such as pregnancy rate per treatment cycle, but also all documents referring to:

1. Control of quality records
2. Internal audits
3. Control of non-conformity
4. Corrective and preventive action

Performance of key indicators is essential, and an example of this in the laboratory is equipment. Incubators are one of the most important pieces of equipment in the IVF laboratory and they need to be controlled properly. Two markers of incubator performance are the temperature and the CO₂ level. These two parameters are documented on the control cards, and upper and lower limits of tolerance are defined to determine when corrective actions are needed (Figure 32.5). It is useful to plot results of system checks on a graph, so that there is a clear visual image that can monitor:

1. Dispersion: increased frequency of both high and low numbers
2. Trend: progressive drift of reported values from a prior mean
3. Shift: an abrupt change from the established mean

If non-conformity to the standard is diagnosed, it is important to collect data on:

1. When the problem was realized
2. How often the problem could be identified
3. How conformity to the standards could be reassured

Audits and management reviews

Audits are essential to ensuring that a quality system is working. Audits can be internal, initiated by the organization itself, or external, initiated by a governing body, certification body, or accreditation body. ISO 9001:2015 lays out the rules for internal audits and demands that a clinic undertakes internal audits at planned intervals to determine how well the system is functioning and if it is effectively implemented and maintained. Audits are tools for improving and keeping your system up to date with the standards. The quality manual should include specific instructions covering both how and how often audits should be

performed. Management usually chooses internal auditors, and they should be familiar with both the standards and the activities performed in the clinic; auditors are from other departments within the organization. The manual should include a document describing the approach and the areas of responsibility for the internal auditors and have well-documented procedures for how internal auditors are trained. To achieve a certification according to ISO 9001:2015, the clinic needs to be audited externally by a certification body. Many organizations believe that having an audit and not finding any non-conformity is proof of outstanding performance. However, the other possibility is that it could be due to an inadequate audit procedure. If an audit is properly conducted, even in organizations with outstanding performance, areas for improvement will be found; therefore, people should put in a lot of effort towards finding the right certification body to undertake the audits. Some questions that might help to identify a good certification body are:

- Are they accredited to certify medical institutions?
- Have they previously certified medical or IVF clinics and how many?
- Do they have medical or IVF experts on their audit team?
- How much time do they allocate to the audit?

Although to some it may seem obvious, it is important to mention, especially with respect to the preceding factors, that the cheapest certifying body is not necessarily the best.

Together with the audits, the management review is important for improvement of the system and for the long-term correction of errors and incidents that might occur. According to ISO 9001:2015 5.6, the management of the clinic with executive responsibility shall periodically conduct a review of the quality system and testing activities. The quality manual shall include a written agenda for these reviews, which should fulfil the demands in the standard.

Incidents and complaints

All clinics should have a policy and procedure for the resolution of incidents and complaints received from patients, clients, or other parties. The routines of how these are filed and how corrective actions are taken should be documented in a clinic's quality manual. When applying a quality system, it is important not to hide these incidents and complaints, but to use them as resources to improve the system. The management reviews should ensure

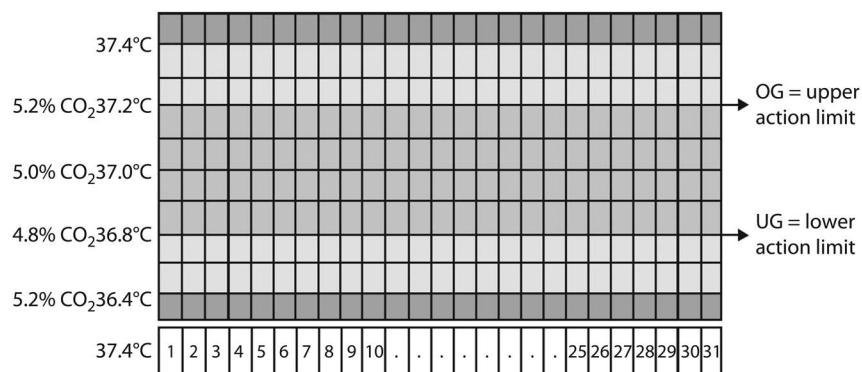


FIGURE 32.5 Monitoring temperature and CO₂ levels in an incubator.

that the incidents and complaints lead to long-term corrections and improvements in the quality of work.

An important part of any QMS is keeping track of "non-conformances." Non-conformances refer to any time an error or event occurs that is not in keeping with procedures in place. In ART, this can be as simple as an incorrect date of birth on a form, or as extreme as an error of using the wrong sperm sample for insemination. It is recommended that a database be established to keep track of non-conformances. It is helpful if the non-conformances are categorized as mild/moderate, significant (e.g. failure to perform ICSI when indicated), or extreme (e.g. transferring the wrong embryo). The frequency of errors should be tracked and a plan for their analysis be in place. Fortunately, serious laboratory errors are infrequent and 99.77% of all procedures are error free [13].

Staff management

High-quality treatment can only be realized with qualified staff. Therefore, recruitment, training, and motivation of highly qualified people are the most important tasks for the management team of an organization. To make sure that a sufficient number of qualified people are working within the respective areas of the institution, a staff requirement plan should be developed. This can be organized in different ways:

1. Allocating people according to their abilities
2. Allocating people according to different responsibility levels
3. Allocating people according to the type of work that has to be done

Medical facilities need to define staffing levels for the different departments in the organization. This is a key role for management, since staffing influences the quality of the service and also the cost-effectiveness/profitability of the organization. The number of employees should be carefully determined for particular departments according to their tasks and the range of services provided. Proactive staff planning where everyone understands his or her role allows for quality service to be delivered. The development of work descriptions is crucial for this system. They must be created for every position, and must clearly state the qualifications and attributes required for the employee. In addition to this formal information, the work description should also contain information about the employee's personal attributes. For various posts, different qualities are important:

1. Social competence
2. Organizing abilities
3. Communication abilities, etc.

The staff requirement plan must be set up so that it is possible to react effectively to unexpected situations. Furthermore, it must consider staff absenteeism caused by holidays, illness, and further education. A minimal presence of employees must be determined for certain areas, irrespective of the actual workload. For the development of a staff requirement plan for an IVF centre, the medical as well as the non-medical areas have to be defined and considered. The question of how many people are needed to do a job properly can be answered on the basis of calculating the "influence magnitudes." The type of services offered strongly influences the number of people required. Thus, the staff

requirements are different in a centre in which predominantly conservative treatments and intrauterine inseminations are performed, compared with a centre in which predominantly IVF and cryopreservation cycles are performed.

Training of employees

One of the most important principles for the management of a medical institution is: "give your employees the chance to be the best." This means that if you expect your employees to do their work at the highest quality level possible, you should give them proper training. In principle, there are two different types of educational events:

1. Internal events of further education
2. External events of further education (i.e. conventions, conferences, workshops, etc.)

The advantage of internal events of further education is that they can be offered on a regular basis and are usually "low-budget projects," whereas external events need more organizational and financial input. However, when carefully planned, external educational events sometimes have a higher motivational aspect. So the management team should take care to offer a balanced program of internal and external educational events. To make it possible to use the clinics' resources adequately, educational and training requirements for the organizational needs should be evaluated on an ongoing basis since unexpected events (e.g. loss of a key employee) can occur. For example, at the beginning of each year, the employee should decide which educational events he or she would like to visit or take part in. This helps the management to introduce new educational opportunities, and also allows them to perform advanced planning of the specialization. It is striking to see that, in most ART centres, detailed and prospective plans have been developed for the training of medical doctors but far less attention has been paid to the training of nurses, technicians, and so on. However, a well-trained nurse can significantly reduce the workload for the doctor and tremendously increase the patient's trust in the institution while also improving the referring doctor's satisfaction.

Therefore, besides training activities for the doctors, adequate educational events for nurses, technicians, and so on should be considered.

Interactions between management and employees

Success in reproductive medicine clearly depends on an optimal interaction between different professional groups; in other words, success can be achieved only if doctors communicate and work together with staff in the laboratory, nurses, receptionists, and so on. The same is true for the interactions between management and employees. Communication and collaboration between different professional groups of the same hierarchic rank is called "horizontal" communication, whereas communication and collaboration between professional groups of different hierarchic ranks is called "vertical" communication. One of the most important instruments for optimizing vertical communication is a staff interview. These staff interviews should occur periodically where employees and their direct supervisors discuss their collaboration and identify areas for improvement. The interview should take place in a structured way and a protocol should be written and signed by both sides, so that the content of the interview is assigned some kind of formal character. However, details of the interview can never be communicated with others without

mutual consent. For the employee, the goals/opportunities of the interview are:

1. To become familiar with the goals of the department
2. To realize weaknesses and strengths
3. To be able to discuss own experiences of/opinions on the management style
4. To discuss further strategies for professional development
5. To participate in planning goals/strategies for the future

For the supervisor, the goals/opportunities of the interview are:

1. To discuss the co-worker's performance
2. To focus the activities of the employee on future goals of the institution
3. To increase mutual understanding in the event of problems
4. To increase the employee's responsibility
5. To get feedback on his/her management skills

For the aforementioned reasons, the staff interview is one of the most important and powerful tools in staff development, and should be widely used in the process of continuous improvement.

The EU Tissue and Cells Directive

The increase in use, donation, and storage of human tissue has led to the creation of directives from the European Council. In March 2004, the European parliament issued a revised version of the directive on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells. When these directives were issued, there was a need to adapt the requirements to the actual setting of an IVF laboratory. However, in the meantime, these directives have been implemented by many IVF centres around the world, and a position paper has been issued by the ESHRE outlining how these directives should be applied. Independent of this position, authorities in many countries interpret the directive differently, which makes it difficult to share experiences between centres in different countries. Furthermore, auditing processes need to be adapted from country to country.

The central part of the EU directive is very clear concerning the demand for a quality system. Therefore, the directive states, "Tissue establishments shall take all necessary measures to ensure that the quality system includes at least the following documentation: standard operating procedures, guidelines, training and reference manuals." Certainly, by achieving ISO accreditation, this demand will be fulfilled, together with several other demands of the directive.

Conclusions

No internationally accepted standards exist for quality in the IVF laboratory and the IVF centre as a whole. To ensure high quality and continual improvement, it is recommended that all IVF centres striving for excellence should consider a QMS. Furthermore, legal guidelines and the EU Tissue and Cells Directive clearly demand a QMS for medical institutions. A QMS allows an organization to gain control of its documents and procedures and to monitor the clinical and non-clinical outcomes. Furthermore, the issues of staff recruitment and staff development can be addressed systematically and the overall outcome will be improved. The ISO standards offer a medical facility access to an internationally

endorsed and proven QMS. ART practitioners in particular have the unique opportunity of setting the standard in medicine for QM principles.

Suggested readings

- Alper MM, Brinsden PR, Fischer R, Wikland M. Is your IVF programme good? *Hum Reprod.* 2002;17:8–10.
- Alper MM. Experience with ISO quality control in assisted reproductive technology. *Fertil Steril.* 2013;100:1503–8.
- American Society for Reproductive Medicine. Revised guidelines for human embryology and andrology laboratories. *Fertil Steril.* 2008;90:s45–59.
- Bloor G. Organisational culture, organisational learning and total quality management: A literature review and synthesis. *Aust Health Rev.* 1999;22:162–79.
- Bron MS, Salmon JW. Infertility services and managed care. *Am J Manag Care.* 1998;4:715–20.
- Brown RW. Errors in medicine. *J Qual Clin Pract.* 1997;17:21–5.
- Carson BE, Alper MM, Keck C. *Quality Management Systems for Assisted Reproductive Technology.* London, U.K.: Taylor and Francis, 2004.
- Clancy C. AHRQ: Coordinating a quantity of quality. *Healthplan.* 2003;44:42–6.
- Collings J. An international survey of the health economics of IVF and ICSI. *Hum Reprod Update.* 2002;8:265–77.
- Colton D. The design of evaluations for continuous quality improvement. *Eval Health Prof.* 1997;20:265–85.
- Darr K. Risk management and quality improvement: Together at last—Part 2. *Hosp Top.* 1999;77:29–35.
- Garceau L, Henderson J, Davis LJ, et al. Economic implications of assisted reproductive techniques: A systematic review. *Hum Reprod.* 2002;17:3090–109.
- Geraedts HP, Montenarie R, Van Rijk PP. The benefits of total quality management. *Comput Med Imaging Graph.* 2001;25:217–20.
- Glattacker M, Jackel WH. Evaluation of quality assurance—Current data and consequences for research. *Gesundheitswesen.* 2007;69:277–83.
- Gondringer NS. Benchmarking: Friend or foe. *AANAJ.* 1997;65:335–6.
- Greenberg L. Accreditation strengthens the disease management bridge over the quality chasm. *Dis Manag.* 2003;6:3–8.
- ISO/DIS 15189:2012. Medical Laboratories—Particular Requirements for Quality and Competence. Geneva, Switzerland: International Standardization Organization, 2012.
- Matson PL. Internal quality control and external quality assurance in the IVF laboratory. *Hum Reprod.* 1998;13(Suppl 4):156–65.
- Minkman M, Ahaus K, Huijsman R. Performance improvement based on integrated quality management models: What evidence do we have? A systematic literature review. *Int J Qual Health Care.* 2007;19:90–104.
- Sackett DL, Rosenberg WMC, Gray JAM, et al. Evidence based medicine: What it is and what it isn't. *Br Med J.* 1996;312:71–6.
- Sakkas D, Barrett CB, Alper MM. Types and frequency of non-conformances in the IVF laboratory. *Human Reprod.* 2018;33:2196–204.
- Sandle LN. The management of external quality assurance. *J Clin Pathol.* 2005;58:141–4.
- Sciacovelli L, Seccihero S, Zardo L, et al. Risk management in laboratory medicine: Quality assurance programs and professional competence. *Clin Chem Lab Med.* 2007;45:756–65.
- Shaw CD. External quality mechanisms for health care: Summary of the ExPeRT project on visitatiae, accreditations, EFQM and ISO assessment in European Union countries. External peer review techniques. European Foundation for Quality Management. International Organization for Standardization. *Int J Qual Health Care.* 2000; 12: 169–75.
- Varkey P, Reller MK, Resar RK. Basics of quality improvement in health care. *Mayo Clin Proc.* 2007;82:735–9.

- Vogelsang J. Quantitative research versus quality assurance, quality improvement total quality management and continuous quality improvement. *J Perianesth Nurs.* 1999;14:78–81.
- Warnes GM, Norman RJ. Quality management systems in ART: Are they really needed? An Australian clinic's experience. *Best Pract Res Clin Obstet Gynaecol.* 2007;21:41–55.
- Yasin MM, Meacham KA, Alavi J. The status of TQM in healthcare. *Health Mark Q.* 1998;15:61–84.

Relevant internet addresses

<http://www.agrbm.de>
<http://www.asrm.com>
<http://www.eshre.com>
<http://www.ferti.net>
<http://www.iso.ch>
<http://www.isoeeasy.org>
<http://www.praxion.com>

References

1. Pinter E, Vitt KD. Umfassendes Qualitätsmanagement für das Krankenhaus—Perspektiven und Beispiele. Frankfurt, Germany: pmi-Verlag, 1996.
2. Viethen G. Qualität im Krankenhaus. Grundbegriffe und Modelle des Qualitätsmanagements. Stuttgart, Germany: Schattauer-Verlag, 1995.
3. ISO 9001:2015. Quality Management Systems—Requirements. Geneva, Switzerland: International Standardization Organization, 2015.
4. ISO/IEC 17025:2005. General Requirements for the Competence of Testing and Calibration Laboratories. Geneva, Switzerland: International Standardization Organization, 2005.
5. ISO/IEC Guide 25. General Requirements for the Competence of Testing and Calibration Laboratories. Geneva, Switzerland: International Standardization Organization, 2005.
6. EN 45001:2017. *General Criteria for the Operation of Testing Laboratories.* www.iso.org
7. American Fertility Society. Revised guidelines for human embryology and andrology laboratories. *Fertil Steril.* 2008;90(3): s45–59.
8. Gianaroli L, Plachot M, Van Kooij R, Committee of the special interest group on embryology, et al. ESHRE guidelines for good practice in IVF laboratories. *Hum Reprod.* 2000;15:2241–6.
9. College of American Pathologists. 2022. Available from: <http://cap.org>
10. Association of Clinical Embryologists UK. Accreditation standards and guidelines for IVF laboratories, 2001. Available from: www.embryologists.org.uk
11. Directive 2004/23/EC of the European Parliament and of the Council of 31 March, 2004. On setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. OJEU. 2004;L102:48–58. Available from: www.eumonitor.nl
12. Huismann W. Quality system in the medical laboratory: The role of a quality manual. *Ann Biol Clin (Paris).* 1994;52:457–61.
13. Sakkas D, Barrett CB, Alper MM. Types and frequency of nonconformances in the IVF laboratory. *Human Reprod.* 2018;33: 2196–204.

Introduction

Remote access to healthcare was accelerated by the Covid-19 global pandemic. Telehealth, work from home, and electronic means to order tests and send prescriptions became necessities. We developed human connections with patients and colleagues in the remote world overnight, with the phrase “you are on mute” part of our daily lives.

With increased digital literacy and enablers such as cloud computing and blockchain comes the possibility to transform both the clinician and patient experience. As server-based legacy systems disappear, they are replaced with cloud computing allowing for high-speed transmission of information through 5G internet with high capacity and security that support integrations with other applications and the internet of things (IoT).

Just as smartphone adoption transformed banking, secure patient portal communication with the IVF clinic will be an expectation from the patients of tomorrow. It seems likely the smartphone is destined to be a digital healthcare enabler. Access to big data via IVF databases will lead to inferences gained through artificial intelligence (AI). This will accelerate the pace of research, inform clinician decision-making, and improve patient outcomes [1].

This chapter examines the advancement of digital technologies which will lead to increased interactions with the IVF clinic, online, in real time. Digitalization will not cease face-to-face interactions but rather improve the accessibility and utility of information. The digital clinic will allow for the provision of precision medicine, tailoring individualized advice and treatment options to patients. This will enhance the experience of fertility care for both patients and providers alike.

Predictive analytics and decision support tools

Real-time decision support via an integrated electronic medical record will allow AI-supported, predictive analytic tools to guide clinician decision-making. The emergence of AI has been described as the “4th industrial revolution” and, in combination with human intelligence, its effect will be transformative to fertility care [2].

AI can mimic human intelligence, and machine learning gives computers the ability to learn without being specifically programmed. Learning from experience, via human-like neural networks, deep learning results in modifications in algorithms to improve performance. The “volume, velocity and variety” of big data is difficult to handle with traditional data analytics [3]. The electronic medical record powered by AI has the potential to cross reference pre-treatment patient characteristics such as age, weight, BMI, AMH, etc., to clinical and laboratory outcomes. The emergence of high-speed internet and cloud computing simultaneously will see the power of AI realized [4].

As databases innovate, data accessibility improves. This not only enhances the ability to perform research, but the insights gained may lead to the development of decision support tools to assist both the doctor and the patient. AI-optimized inference tools could guide the doctor and the patient as to whether to continue or cancel an IVF cycle [5]. The potential of decision-support tools has been explored in treatment option choice for the management of ectopic pregnancy [6]. Decision tools to determine the optimal day for trigger injection to optimize laboratory outcomes has already been demonstrated [7]. Guidance on the best dose of FSH, cycle type, whether to use an agonist trigger to prevent OHSS will become more predictive with large data sets.

In dermatology, when integrated with the electronic medical record (EMR), the ability to combine clinical data such as previous skin cancer history and demographics with imaging data and histological diagnosis to inform prognosis is realized [8]. In the field of reproductive medicine, it is conceivable that an individualized prediction of live birth could be ascertained and updated with each treatment stage; before, during, and after treatment, based on all the available data points, such as age, cause of infertility, AMH level, follicular size, quantitative HCG, and embryo quality [9]. In addition, individualized pregnancy rates could provide more price certainty around the cost of achieving a live birth as well as identify the most economical treatment options available to the patient.

With the rapid pace of computer and genomic science, the future of medicine is likely to see a personalized digital fingerprint or “digitome” embedded into a patient’s medical record, integrating genomic, epigenomic, metabolomic, transcriptomic, proteomic, microbiomic, and pharmacogenomic data together with patient history, demographics, imaging, test results, and environmental exposures. Interestingly, the Oxford Nanopore sequencing platform uses miniaturized DNA sequencing technology and has achieved comprehensive DNA sequence analysis using a smartphone [10]. The ability to capture and utilize AI to interpret these data points will revolutionize the concept of precision medicine.

With regards to pharmacogenomics, the genetic heterogeneity between each IVF patient is an opportunity to tailor IVF treatment specifically for that individual. For example, FSH receptor polymorphisms are known to contribute to variable response to IVF stimulation, and specifically targeted drug treatment could improve response [11]. Integration of pharmacogenomic decision support into the clinical workflow has been explored and requires systems support with a genetic data repository, interpretation of biomarkers and return of a report to the clinical information system [12].

The power of bioinformatics will be needed to manage the vast amounts of data that genome-wide sequencing can generate [13]. Correlation of phenotype with genotype is relatively straightforward for Mendelian conditions but more challenging when faced with variants of unknown significance. Data sharing can assist in

establishing the significance of a new variation. In the emerging field of polygenic risk scores, predictions can be made regarding cardiovascular or cancer risk for an individual, although caution is required in their interpretation [14]. Interestingly, facial recognition can assist the geneticist in the diagnosis of rare genetic syndromes, correlating genotype to phenotype utilizing a clinical software package known as Face2Gene [15]. Optimal management of genomic data will be essential to facilitate its clinical utility.

The development of clinician decision support systems will require data sets from a diverse patient population to gain insights. Large, interconnected IVF clinics with data sharing will have an advantage over smaller clinics. The ability to embed these tools into the clinical workflow of the digital database should improve cycle outcomes. However, the “black box” nature of AI will be hypothesis generating rather than hypothesis driven and will require doctors to interpret how these predictions were established and critically evaluate for flaws and biases in the machine models [16, 17]. Clinician input and validation will be essential to ensure the relevance and utility of these predictive analytic tools [18]. Regulatory requirements will be necessary with the introduction of new AI tools to ensure safety and efficacy where conclusions lack “explainability”, however ultimately AI will enhance the decision-making of the doctor [19].

Enhanced clinician experience

A single, integrated view of a person’s treatment and harmonized clinical workflows allow for efficient information sharing amongst the medical care team. This improved workforce experience reduces the time spent by clinicians on administration activities and reduces the potential for paper-based errors. For example, medication errors can be reduced by transitioning from paper-based to electronic prescribing [20]. The electronic transmission of an encrypted QR code to a patient’s mobile device allows them to present this at their local pharmacist to scan the “token” and collect their medication. The ability to confirm IVF treatment plans from treatment templates allows for seamless transmission of treatment instructions via a patient portal that also populates the activity requirements into the laboratory workflow.

Data management is an essential part of quality management, and paperless systems minimize the risk of transcription errors and privacy breaches, e.g. where incorrect paperwork is given to the wrong patient. Where paper is received from external sources, automatic extraction of useful information and AI-assisted auto-population of results, even from unstructured documents with varied layouts, are opportunities for the future [21].

A digital system with a rich core functionality user interface can leverage the benefits to all job types within the clinic. Speech recognition dictation could be utilized to summarize patient appointments, dictate progress notes, and even transcribe video-recorded patient consults. Interoperability with IoT provides endless opportunities to integrate applications facilitated by cloud-based technologies as opposed to server. For example, connection to the IoT with such technology as ultrasound machines may make it possible to capture follicle ultrasound images directly into the digital database, with images being transmitted using DICOM standards of encryption (Digital Imaging and Communications in Medicine) [22]. Reduction in inter-observer error through the use of automated 3D follicle measurement and the use of home transvaginal ultrasound devices that transmit

data via the smartphone to the clinic will revolutionize how the IVF clinic of the future would run [23].

Personalized feedback to clinicians on their performance, with respect to pregnancy rates, could be evaluated using more specific subgroups of patients, allowing insights into why some doctors have higher live birth rates than others. In addition, an optimized digital fertility clinic would provide integration for data sharing and reporting to national registries, ideally automatically.

The digital fertility clinic of the future would incorporate interactive clinician education and summarize current research. A fertility specialist would need to read for 29 hours a day to keep on top of new research. Analysing scientific literature through AI has provided assistance to fields like oncology, using cognitive computers to determine optimal therapeutic approach based on tumour characteristics [24]. Providing an AI-assisted summary of the latest scientific literature will assist the clinician and allow for best practice medical care.

Optimize clinic workflow

The inverse relationship between high volume workload and reduced outcomes is well recognized. By linking the predicted number of oocytes to a scheduling algorithm, it is possible to even out workflow during the week [5]. Predicting workload would allow clinical teams and IVF laboratories to optimize their staffing. AI-driven rostering, taking into account a set of constraints such as staff availability and preferences, working hour regulations, clinic requirements, and anticipated workload, can ultimately improve efficiency.

Analytical tools have been used to predict absenteeism and then evaluate the effectiveness of interventions, such as stress management programs, to reduce levels [25]. Other human resource applications are screening of resumes to recruit and select new employees and in performance management [26].

Robotic process automation for repetitive tasks such as updating patient registration and billing are achievable. Automation of proof of identity processes where patients upload documents and utilization of facial recognition can replace manual administrative tasks depending on business rules. AI assistance for supply chain management with monitoring of stock via barcodes and sensors and automatic ordering based on anticipated workload will provide efficiencies [27].

Digitizing the IVF laboratory

Other areas of medicine such as radiology and microbiology are seeing the successful incorporation of digital technologies into their workflows. AI interpretation of imaging is approaching diagnostic equivalence to a radiologist, assisting in the triage of large volumes of imaging [28]. Utility has been found in microbiology to count bacterial colonies and identify pathogenic species [29]. Equally, use of digital pathology allows for AI-based algorithms capable of capturing subtle patterns in tumour development beyond human recognition [30]. An extension of this is computational pathology, combining tumour imaging and genomic features, which will require integration of laboratory information systems (LIMS) and imaging management systems in order to be accessible for clinical use [31].

Most clinics around the world today are still reliant on paper as their form of data capture [32]. Important information such as Pre-implantation genetic testing (PGT) results are recorded in separate documents and time-lapse imaging is rarely integrated

into the patient management system. By not linking this data to their biological items, the opportunity to gain insights into the data is compromised. The removal of the reliance of paper-based workflows and transition to digitalization of the IVF clinic will be an essential step towards improvement of patient outcomes.

Connecting biomedical devices to the fertility database allows the data gained via continuous monitoring of the laboratory environment to be integrated and utilized. This provides an ability to transmit alerts via notifications to multiple devices, including smartphones, when deviations occur in important quality control (QC) parameters such as CO₂, pH, O₂, liquid nitrogen level, VOCs, temperature, and relative humidity. In addition, door sensors can monitor for security breaches, or vibration detectors can detect environmental events. This would permit immediate actions to be taken by staff, even if off-site, optimizing safety of the IVF laboratory.

Continuous analysis of laboratory **key performance indicators** (KPIs) covering a range of parameters, such as fertilization rate, embryo utilization rate, and clinical pregnancy rate, would be facilitated by a digital database. When data is available in real time, shifts in performance can readily be detected, allowing for a contemporaneous response to problem solving [33]. Transition to the digital laboratory would allow for real-time feedback for the embryologist considering multiple variables, such as treatment types, stimulation protocol, medications, patient age, and clinic location, and cover a range of embryological parameters. Performance appraisal of the embryologist via a range of KPIs would be possible.

To improve performance, interactive 360-degree virtual environments for staff training, linking educational material to the virtual laboratory, have been shown to increase engagement and motivation leading to improved learning [34]. Utilizing these technologies to engage staff in ongoing education and execution of work instructions in the laboratory will improve safety.

The tracking and tracing of biological items is an essential component to risk minimization in the IVF clinic. Electronic witnessing systems have been demonstrated to reduce the chance of a mix-up in eggs, sperm, and embryos [35]. An extension of this technology is the development of radio frequency identification (RFID) tags, now capable of working at cryogenic temperatures, thereby reducing the risk that the incorrect gametes or embryos are used in a treatment cycle [36]. By integrating witnessing information to the patient record, it would be possible to perform full audits of biological items in storage and address the statutory requirements with regards to the length of time biological items have been stored.

Intelligent computer systems that allow automation of some laboratory processes may assist in reducing the number of repetitive tasks performed by the embryologist [37, 38]. We will witness a decrease in the manual manipulation of eggs, sperm, and embryos through automation. In the andrology laboratory, there is a gradual shift away from manual semen analysis and towards automation with increasing capability to optimize assessment via AI [39]. "Lab on a chip" microfluidic devices with chemotaxis and imaging capabilities may prove to be an ideal sperm-sorting method for sperm preparation in the automated IVF laboratory [40].

Further reduction in manual handling of gametes may be seen via imaging to assess oocyte competency prior to intracytoplasmic sperm injection (ICSI) as well as to determine normal fertilization [41]. The development of micro-robotic ICSI will require optimization of imaging processing algorithms that can confirm

successful oolemma penetration before sperm injection before becoming a reality [42, 43]. Improving efficiency through digital automation and the capability of remote observations will allow flexible working hours, which is particularly beneficial where highly trained embryologists are in short supply. Time-lapse incubators and AI assistance allow monitoring embryos undisturbed, and selection of the best for transfer could be attained with staff working remotely [44].

It is well recognized that any stress encountered within the embryo culture system can reduce the ability of an embryo to implant [45]. Traditional research examines just a few of these variables on pregnancy outcome in one study, whereas the birth of a healthy baby results from the interaction of multiple variables both inside and outside the laboratory and is unique to that individual. For example, one such variable within the culture system known to be consumed in large amounts by embryos most likely to lead to pregnancy is glucose [46]. However, the *in vivo* environment is dynamic and contains many substances in varied amounts at different times. The monitoring of this environment as well as the delivery of embryo trophic factors could be achieved through these micro-perfusion technologies. To create and monitor the stage-specific requirements for an embryo would require 3D microfabrication of an embryo chamber capable of micro-perfusion. In addition to mirroring the *in vivo* environment, this would assist in the non-invasive assessment embryo viability via analysis of its "secretome" obtained from spent media. The insights gained through non-invasive metabolic and genomic assessments could lead to the development of an "embryo health score" to assist in clinical decision-making [47].

Outside the laboratory, the persons providing the egg and sperm have a unique fertility profile, including factors such as previous pregnancies, BMI, and smoking status that will contribute to the IVF outcome [48]. All this information would require effective data capture, in real time, to support the IVF laboratory of tomorrow. To gain meaningful insights into the multitude of variables leading to the birth of a healthy baby could only be realized with a digitalized IVF laboratory and the power of AI.

Changing the way patients communicate with the IVF clinic

Digital solutions that take patient care outside of the fertility clinic will change the way we provide healthcare for the better. Many patients currently source health information from the internet, and the expectation of secure, smartphone communication with their clinic is emerging. Education empowers patients to make their own healthcare decisions. Digital solutions allow distribution of information to a wide audience, with the potential to democratize access to fertility education. In addition, we need to deliver person-centred information that is individualized for that patient and delivered at the right time. For example, precision medicine in the fertility clinic of the future would lay out the personalized likelihood of success, time to pregnancy, and the estimated cost for each treatment option. These individualized treatment plans would take into account the fertility profile of the egg and sperm provider, environmental exposures, genomics, and diagnostic tests results. In addition, alteration of the outcomes could be demonstrated if modification to variables changed, such as weight loss, or if additional interventions, such as meditation or stress management strategies, were undertaken. Such AI-assisted decision support tools provide the possibility of

scalability of patient-centred solutions which would transform the way fertility care is delivered.

When patients are educated, they proactively participate in their own well-being and have better health outcomes. Each person learns differently, and the delivery of educational material in a variety of forms, such as verbal, written, video, and interactive, improves patients' understanding. Electronic consenting software supported by video content has been shown to improve patient satisfaction of the consent process and understanding of IVF procedures [49, 50].

Making access to fertility care easier can be facilitated with online appointment bookings, virtual consults, electronic prescriptions, pathology, and imaging requests digitally signed by the doctor. Online ordering and home delivery of medication, vitamins, and ovulation kits as well as virtually assisted injection instruction can reduce the time burden of attending clinics. Reminders for when patients should take their injections and secure electronic messaging with the clinic for questions would be possible in a patient-centred digital clinic.

Access to real-time laboratory data, such as embryo, egg, and sperm imaging, as well as the ability to share to social media, are opportunities to engage patients. Rules around when and how critical information, such as low fertilization results, is provided to patients would need to be carefully considered.

The advances in natural language processing may lead in the future to the analysis of clinical notes, the ability to prepare reports and engage in conversational AI. Chatbot for common questions is possible due to natural language-processing technology and is commonly used on websites in other domains. Responses may provide answers to simple questions that would reduce the time requirements for clinic staff and make the clinic available 24 hours a day. These tools would require appropriate validation in terms of accuracy of comprehension and information and user experience [51].

Feedback and mood-tracking capabilities can be captured in real time in a digital clinic. Social robots could be integrated that could assess mood, give emotional support, and possibly even provide treatment in the form of cognitive behavioural therapy [52]. AI deep learning models have been shown to sense and respond to emotion [53]. Automatic speech recognition and real-time language translation with the capacity for speech-to-text translation or use of subtitles may reduce language barriers and the need for translators to turn up to the clinic in the future [54]. Complementary services such as online counselling, group counselling, access to dieticians and online communities are all potential features of the digital IVF clinic of tomorrow.

A digital IVF clinic that includes an online donor bank for gametes and embryos and search functions for donor characteristics would give patients the ability to make these decisions from home. The ability to seamlessly send information between clinics and provide patients with options for what to do with their excess eggs, sperm, and embryos could improve the patient experience.

New ways to gather and interpret data

Widespread smartphone use has resulted in the potential to utilize patient-generated data from wearables, for example vital signs, ovulation tracking, survey data, and mental health checklists.

Wrist wearables that capture the biphasic pattern of skin temperature during the menstrual cycle and measurement of night-time body temperature can retrospectively confirm ovulation

[55]. Bluetooth communication of urinary luteinizing hormone results and downloading this information to fertility apps are currently used by patients [55]. Incorporation of this information into the digital fertility clinic remains an opportunity. Smartphone-based analytics could mean the standard one or two points in time semen analysis could change to repeated measurements performed in the comfort of the home and a different view of normality for male fertility [56].

Wearables can track other digital biomarkers, such as activity level, heart rate, and sleep. Deep learning algorithms can use these biomarkers to determine sleep quality and make inferences about melatonin onset and circadian rhythms [57]. Incorporating this information into the fertility database would allow further evaluation of past observations that disrupted sleep is associated poor reproductive outcomes [58, 59]. There would be an opportunity for patients to become aware of their sleep patterns and address them with behavioural modification.

Electrochemical sensors that are capable of measuring sweat for the stress hormone cortisol are advancing [60, 61], as are continuous glucose monitors [62]. Similarly, applications are being found in other areas of medicine. In ophthalmology, a hand-held device with AI-assisted image analysis to screen for diabetic retinopathy holds promise to offer diagnostics to remote regions [63]. Wearables such as the Apple watch can detect atrial fibrillation and alert emergency services [64]. Although there is potential for wearables and their associated apps to encourage healthy living, well-being, and improve healthcare delivery, there is a need for ongoing review of regulation. For example, independent evaluation of algorithm-based apps for the assessment of skin lesions, showed there is little evidence supporting their use for self-monitoring and detection of skin cancer [65]. The FDA oversight of mobile medical applications informs of the risk to a patient's safety if the mobile app were to not function as intended [66]. Further evaluation of subsequent generations of such health apps, improved through deep learning, may see the refinement of these technologies.

Mobile health apps may improve healthcare delivery through their reach and scalability and may find their place through supporting the doctor with long-distance, real-time assessment and feedback and future diagnostic innovations [67]. We have the responsibility of ensuring that effective implementation and responsible use of such technologies occurs. Although many of these emerging innovations are still in their infancy, they hold the promise of supporting long-distance healthcare.

Governance, legislation, and design considerations

There is an ever-evolving range of regulatory and legal requirements that govern digital healthcare across countries, which must be considered during production and implementation. Robust governance frameworks are required to address development of architecture, data management, legislation, compliance, innovation, integration with external systems, and cybersecurity [68].

Information must be accessible without compromising patient privacy, particularly when transmitting data between systems. To achieve integration and interoperability with other applications, the digital fertility clinic must manage heterogenous data from multiple sources. The use of blockchain simplifies authentication procedures by omitting intermediaries. This can address the challenges of sharing medical records, interoperability, and IoT

security. In addition to data management, systems must consider what data they will capture, how this will be standardized, and who will have authorized access.

The future of fertility research in areas such as genomics and biomedical devices will be assisted by AI and big data. As legislation attempts to keep pace with AI technology, it must evolve in a way that allows research to progress but also ensures patient safety.

Digital transformation of a fertility clinic is ideally undertaken with key stakeholders and subject matter experts that act as co-creators, mapping the current workflows, identifying alterations, and drafting an implementation roadmap that considers how rollout should occur. Consideration should be given to workforce adaptability with respect to digital literacy, attitude, and training requirements. The professional culture of the organization, trust in technology, accountability, and the identification of staff that can act as enablers is essential.

Conclusion

The next generation of IVF clinics will be digitalized and patient-centric, providing a superior experience for both patients and providers of fertility care. Data capture in real time will improve the transmission and utilization of information. Accessibility to data will increase and will extend to digital biomarkers captured by wearables, integration with IoT, and partnering with external data sources. Patients will have a unique digital footprint. Big data in combination with AI and mediated by human intelligence will allow meaningful insights that can be translated into clinical decision-making tools, leading to predictive and personalized treatment options. This precision fertility will revolutionize how we deliver care and will improve outcomes for our patients.

References

- Alexander CA, Wang L. Big data and data-driven healthcare systems. *J Bus Manag Sci.* 2018;6(3):104–11.
- Yorks L, Rotatori D, Sung S, Justice S. Workplace reflection in the age of AI: Materiality, technology, and machines. *Adv Dev Hum Resour.* 2020;22(3):308–19.
- Manogaran G, Varatharajan R, Lopez D, Kumar PM, Sundarasekar R, Thota C. A new architecture of internet of things and big data ecosystem for secured smart healthcare monitoring and alerting system. *Future Gener.* 2018;82:375–87.
- Davenport T, Kalakota R. The potential for artificial intelligence in healthcare. *Future Healthc J.* 2019;6(2):94–8.
- Letterie G, Mac Donald A. Artificial intelligence in in vitro fertilization: A computer decision support system for day-to-day management of ovarian stimulation during in vitro fertilization. *Fertil Steril.* 2020;114(5):1026–31.
- De Ramón Fernández A, Ruiz Fernández D, Prieto Sánchez MT. A decision support system for predicting the treatment of ectopic pregnancies. *Int J Med Inform.* 2019;129:198–204.
- Hariton E, Chi EA, Chi G, Morris JR, Braatz J, Rajpurkar P, et al. A machine learning algorithm can optimize the day of trigger to improve in vitro fertilization outcomes. *Fertil Steril.* 2021;116(5):1227–35.
- Du-Harpur X, Watt FM, Luscombe NM, Lynch MD. What is AI? Applications of artificial intelligence to dermatology. *Br J Dermatol.* 2020;183(3):423–30.
- Goyal A, Kuchana M, Ayyagari KPR. Machine learning predicts live-birth occurrence before in-vitro fertilization treatment. *Sci Rep.* 2020;10(1):20925.
- Palatnick A, Zhou B, Ghedin E, Schatz MC. iGenomics: Comprehensive DNA sequence analysis on your smartphone. *GigaScience.* 2020;9(12):1–12.
- Kalinderi K, Asimakopoulos B, Nikolettos N, Manolopoulos VG. Pharmacogenomics in IVF: A new era in the concept of personalized medicine. *Reprod Sci.* 2019;26(10):1313–25.
- Tippenhauer K, Philips M, Largiadèr CR, Sariyar M, Bürkle T. Integrating pharmacogenetic decision support into a clinical information system. *Stud Health Technol Inform.* 2020;270:618–22.
- Capalbo A, Poli M, Riera-Escamilla A, Shukla V, Hoffding MK, Krausz C, et al. Preconception genome medicine: Current state and future perspectives to improve infertility diagnosis and reproductive and health outcomes based on individual genomic data. *Hum Reprod Update.* 2021;27(2):254–79.
- Forzano F, Antonova O, Clarke A, de Wert G, Hentze S, Jamshidi Y, et al. The use of polygenic risk scores in pre-implantation genetic testing: An unproven, unethical practice. *Eur J Hum Genet.* 2022;30(5):493–5.
- Gurovich Y. The path to and impact of disease recognition with AI. *IEEE Pulse.* 2020;11(1):13–6.
- König H, Frank D, Baumann M, Heil R. AI models and the future of genomic research and medicine: True sons of knowledge?: Artificial intelligence needs to be integrated with causal conceptions in biomedicine to harness its societal benefits for the field. *BioEssays.* 2021;43(10):e2100025.
- Raza K, Dey N. *Translational Bioinformatics Applications in Healthcare.* First edition. Boca Raton: CRC Press, 2021.
- Kubben P, Dumontier M, Dekker A. *Fundamentals of Clinical Data Science.* The Netherlands: SpringerOpen, 2019.
- Rajam N. Policy strategies for personalising medicine “in the data moment”. *Health Policy Technol.* 2020;9(3):379–83.
- Ababneh MA, Al-Azzam SI, Alzoubi KH, Rababa'h AM. Medication errors in outpatient pharmacies: Comparison of an electronic and a paper-based prescription system. *J Pharm Health Serv Res.* 2020;11(3):245.
- Dipali B, Swati A, Vidyasagar P, Ketan K. Efficient automated processing of the unstructured documents using artificial intelligence: A systematic literature review and future directions. *IEEE Access.* 2021;9:72894–936.
- Lebre R, Silva LB, Costa C. A cloud-ready architecture for shared medical imaging repository. *J Digit Imaging.* 2020;33(6):1487.
- Gerris JMR, Fauser BCJM (eds.). *Home Monitoring of Ovarian Stimulation: an Important Step towards More Patient-Centred IVF 2020.* Netherlands: Reproductive Healthcare Ltd, 2020.
- Curioni-Fontecedro A. A new era of oncology through artificial intelligence. *ESMO Open.* 2017;2(2):e000198.
- Lawrance N, Petrides G, Guerry M-A. Predicting employee absenteeism for cost effective interventions. *Decis Support Syst.* 2021;147:113539.
- Bhardwaj G, Singh SV, Kumar V. An empirical study of artificial intelligence and its impact on human Resource functions. *IEEE.* 2020;47:51.
- Earley S. The future of supply chain management is AI and data. *SCMR.* 2021;24(1):24.
- Adams SJ, Henderson RDE, Yi X, Babyn P. Artificial intelligence solutions for analysis of x-ray images. *Can Assoc Radiol J.* 2021;72(1):60–72.
- Sharp S. AI advances efficiency in the lab. *MLO: Medical Laboratory Observer.* 2020;52(1):40–1.
- Rakha EA, Toss M, Shiino S, Gamble P, Jaroensri R, Mermel CH, et al. Current and future applications of artificial intelligence in pathology: A clinical perspective. *J Clin Pathol.* 2021;74(7):409–14.
- Cui M, Zhang DY. Artificial intelligence and computational pathology. *Lab Invest.* 2021;101(4):412–22.
- Curchoe CL. The paper chase and the big data arms race. *J Assist Reprod Genet.* 2021;38(7):1613–15.
- Bormann CL, Curchoe CL, Thirumalaraju P, Kanakasabapathy MK, Gupta R, Pooniwala R, et al. Deep learning early warning system for embryo culture conditions and embryologist performance in the ART laboratory. *J Assist Reprod Genet.* 2021;38(7):1641–6.

34. Viitaharju P, Yliniemi K, Nieminen M, Karttunen AJ. Learning experiences from digital laboratory safety training. *Educ Chem Eng.* 2021;34:87–93.
35. Thornhill AR, Brunetti XO, Bird S, Bennett K, Rios LM, Taylor J. Reducing human error in IVF with electronic witnessing. *Fertil Steril.* 2011;96(3):S179–S.
36. Marks P. Cryogenic RFID tags aim to end to IVF mix ups. *New Scientist.* 2009;202(2714):22.
37. Bori L, Meseguer M. Will the introduction of automated ART laboratory systems render the majority of embryologists redundant? *Reprod Biomed Online.* 2021;43(6):979–81.
38. Gardner DK. ‘The way to improve ART outcomes is to introduce more technologies in the laboratory.’ *Reprod Biomed Online.* 2022;44(3):389–92.
39. Agarwal A, Henkel R, Huang C-C, Lee M-S. Automation of human semen analysis using a novel artificial intelligence optical microscopic technology. *Andrologia.* 2019;51(11):e13440.
40. Marzano G, Chiriacò MS, Primiceri E, Dell’Aquila ME, Ramalho-Santos J, Zara V, et al. Sperm selection in assisted reproduction: A review of established methods and cutting-edge possibilities. *Bio technol Adv.* 2020;40:107498.
41. Federica C, Mario Z, Valeria M, Thi Thu Hien B, Martina B, Lorenzo F, et al. A neural network-based identification of developmentally competent or incompetent mouse fully-grown oocytes. *J Vis Exp.* 2018;(133):56668.
42. Mor A, Zhang M, Esencan E, Simsek B, Nichols-Burns SM, Liu Y, et al. A step towards the automation of intracytoplasmic sperm injection: Real time confirmation of mouse and human oocyte penetration and viability by electrical resistance measurement. *Fertil Steril.* 2020;113(1):234–6.
43. Lu Z, Zhang X, Leung C, Esfandiari N, Casper RF, Sun Y. Robotic ICSI (intracytoplasmic sperm injection). *IEEE Trans Biomed Eng.* 2011;58(7):2102–8.
44. Tran D, Cooke S, Illingworth PJ, Gardner DK. Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer. *Hum Reprod.* 2019;34(6):1011–8.
45. Gardner DK, Kelley RL. Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *J Dev Orig Health Dis.* 2017;8(4):418–35.
46. Ferrick L, Lee YSL, Gardner DK. Metabolic activity of human blastocysts correlates with their morphokinetics, morphological grade, KIDSscore and artificial intelligence ranking. *Hum Reprod.* 2020;35(9):2004–16.
47. Ferrick L, Lee YSL, Gardner DK. Reducing time to pregnancy and facilitating the birth of healthy children through functional analysis of embryo physiology†. *Biol Reprod.* 2019;101(6):1124–39.
48. Hornstein MD. Lifestyle and IVF outcomes. *Reprod Sci.* 2016; 23(12):1626–9.
49. Bowers N, Eisenberg E, Montbriand J, Jaskolka J, Roche-Nagle G. Using a multimedia presentation to improve patient understanding and satisfaction with informed consent for minimally invasive vascular procedures. *Surgeon.* 2017;15(1):7–11.
50. Madeira JL, Rehbein J, Christianson MS, Lee M, Parry JP, Pennings G, et al. Using the EngagedMD multimedia platform to improve informed consent for ovulation induction, intrauterine insemination, and in vitro fertilization. *Fertil Steril.* 2018;110(7):1338–46.
51. Abd-Alrazaq A, Safi Z, Alajlani M, Warren J, Househ M, Denecke K. Technical metrics used to evaluate health care chatbots: Scoping review. *J Med Internet Res.* 2020;22(6):e18301.
52. Dino, F., Zandie, R., Abdollahi, H., Schoeder, S., & Mahoor, M.H. (2019). Delivering Cognitive Behavioral Therapy Using A Conversational Social Robot. 2019 IEEE/RSJ International Conference on Intelligent Robots and Systems (IROS), 2089–2095.
53. Day M-Y, Hung C-S. AI affective conversational robot with hybrid generative-based and retrieval-based dialogue models. *IEEE Trans Affective Comput.* 2019;403–9.
54. Chuang S, Liu AH, Sung T, Lee H. Improving automatic speech recognition and speech translation via word embedding prediction. *IEEE/ACM Transactions on Audio, Speech, and Language Processing, Audio, Speech, and Language Processing, IEEE/ACM Transactions on, IEEE/ACM Trans Audio Speech Lang Process.* 2021;29:93–105.
55. Shilah M, Goodale BM, Falco L, Kübler F, De Clerck V, Leeners B Modern fertility awareness methods: Wrist wearables capture the changes in temperature associated with the menstrual cycle. *Biosci Rep.* 2018;38(6):BSR20171279.
56. Tsao YT, Yang CY, Wen YC, Chang TC, Matsuura K, Chen Y, et al. Point-of-care semen analysis of patients with infertility via smartphone and colorimetric paper-based diagnostic device. *Bioeng Transl Med.* 2021;6(1):1–11.
57. Castaldo R, Chappell MJ, Byrne H, Innonimato PF, Hughes S, Pescapè A, et al. Detection of melatonin-onset in real settings via wearable sensors and artificial intelligence. A pilot study. *Biomed Signal Process Control.* 2021;65:102386.
58. Stocker LJ, Cagampang FR, Lu S, Ladyman T, Cheong YC. Is sleep deficit associated with infertility and recurrent pregnancy losses? Results from a prospective cohort study. *Acta Obstetricia et Gynecologica Scandinavica.* 2021;100(2):302.
59. Stocker LJ, Macklon NS, Cheong YC, Bewley SJ. Influence of shift work on early reproductive outcomes: A systematic review and meta-analysis. *Obstet Gynecol.* 2014;124(1):99–110.
60. Zea M, Bellagambi FG, Ben Halima H, Zine N, Jaffrezic-Renault N, Villa R, et al. Electrochemical sensors for cortisol detections: Almost there. *Trends Anal Chem.* 2020;132:116058.
61. Parlak O, Keene ST, Marais A, Curto VF, Salleo A. Molecularly selective nanoporous membrane-based wearable organic electrochemical device for noninvasive cortisol sensing. *Sci Adv.* 2018;4(7):eaar2904.
62. Bent B, Wang K, Grzesiak E, Jiang C, Qi Y, Jiang Y, et al. The digital biomarker discovery pipeline: An open-source software platform for the development of digital biomarkers using mHealth and wearables data. *J Clin Transl Sci.* 2020;5(1):e19.
63. Rogers TW, Gonzalez-Bueno J, Garcia Franco R, Lopez Star E, Méndez Marín D, Vassallo J, et al. Evaluation of an AI system for the detection of diabetic retinopathy from images captured with a handheld portable fundus camera: The MAILOR AI study. *Eye (Lond).* 2021;35(2):632–38.
64. Tison GH, Sanchez JM, Ballinger B, Singh A, Olglin JE, Pletcher MJ, et al. Passive detection of atrial fibrillation using a commercially available smartwatch. *JAMA Cardiol.* 2018;3(5):409–16.
65. Freeman K, Dinnis J, Chuchu N, Takwoingi Y, Bayliss SE, Matin RN, et al. Algorithm based smartphone apps to assess risk of skin cancer in adults: Systematic review of diagnostic accuracy studies. *BMJ.* 2020;368:m127.
66. FDA. Policy for Device Software Functions and Mobile Medical Applications. 2019, <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/policy-device-software-functions-and-mobile-medical-applications>
67. Xiong M, Pfau J, Young AT, Wei ML. Artificial intelligence in tele-dermatology. *Curr Dermatol Rep.* 2019;8(3):85.
68. Aerts A, Bogdan-Martin D. Leveraging data and AI to deliver on the promise of digital health. *Int J Med Inform.* 2021;150:104456.

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LIFESTYLE, PERICONCEPTION, AND FERTILITY

Jessica A. Grieger, Lisa J. Moran, Sarah A. Robertson, Rui Wang, and Robert J. Norman

Introduction

Reproductive health critically impacts well-being and functional capacity throughout life, from adolescence to older age. The majority of women and many men experience some form of reproductive disorder at some time in life, and many chronic and severe reproductive disorders remain without preventative strategies, clear diagnostics, or successful treatment options. Even an apparently “normal” pregnancy can pose a health challenge and reveal or precipitate underlying chronic metabolic disease and/or cardiovascular dysfunction in women. The direct cost of maternal and neonatal conditions to individuals, families, and communities is substantial [1].

Importantly, the reproductive health of a woman and of her partner at the time of conception is the single greatest determinant of the health and well-being of their children [2]. The periconception period in humans is depicted by the five stages of reproductive development: gametogenesis, fertilization, implantation, embryogenesis, and placentation [3]. It is evident that the critical influence of parents begins even before conception, whereby a compromised egg or sperm from either parent can alter the developmental trajectory of a fetus, even if the embryonic development and intrauterine environment are not obviously compromised [2, 4]. Factors in addition to the gametes are also important—the immune response and receptivity of a woman’s uterus can substantially affect the quality of implantation and placental development [5], and this receptivity is impacted by the composition of the male partner’s seminal fluid [6]. A suboptimal environment during fetal development in utero predisposes an individual to diseases in adulthood, including obesity, heart disease, diabetes, and stroke, to an extent comparable in magnitude to genetic predisposition and lifestyle factors such as obesity and smoking [7]. Understanding early life events and how they contribute to health or resilience to disease is a fundamental component of intergenerational health, in that the health of one generation affects that of the next.

Fundamental knowledge gaps that still remain are:

1. What environmental and genetic factors determine the optimal function of sperm and eggs, and facilitate receptivity of the uterus?
2. What are the critical biological events and pathways in the periconception period that promote or constrain developmental competence in the oocyte and embryo to affect health and functional capacity in later life?
3. How do environmental conditions, genes, and maternal reproductive disorders influence developmental competence in the oocyte and embryo, and optimal growth in the fetus?
4. How do we best translate fundamental knowledge gains to better predict and diagnose reproductive disorders, improve periconception health, and maximize pregnancy outcome?
5. What is the role of male factors in determining health in the sperm, embryo, and fetus?

Goals of periconception health

Our goal should be to make important basic science and epidemiological discoveries and to capitalize on these to prevent disease and disability and build resilience in our communities through clinical and public health interventions, targeting early stages in life. This is best achieved by a cross-disciplinary approach that spans basic biomedical science, epidemiology, and translational research. Integration of cell and molecular biology, physiology, immunology, and new technologies (genomics and sensing) with clinical and epidemiological studies promises the best approach to developing new paradigms for appropriate healthcare. Periconception care is more than just improving fertility and ensuring an uncomplicated pregnancy—it is also about optimal outcomes for children born as a result of both natural conception and after assisted reproductive techniques (ART).

Societal importance

The global community recognizes the critical value of reproductive health and its necessity for health and resilience in our children. International commitment to reproductive health was declared at the 1994 International Conference on Population and Development in Cairo [8], reaffirmed at the 1995 Fourth World Conference on Women [9], and reinforced in 2000, when the UN Millennium Declaration specified the 5th Millennium Development Goal to “Improve maternal health,” with a focus on sexual and reproductive health [10, 11]. The Special Programme of Research, Development and Research Training in Human Reproduction (HRP), today a United Nations (UN) Programme, co-sponsored by the United Nations Development Programme (UNDP), the United Nations Population Fund (UNFPA), the World Bank, and the World Health Organization (WHO) are the main instruments within the UN system for research in human reproduction, bringing together policymakers, scientists, healthcare providers, clinicians, consumers, and community representatives to identify and address priorities for research to improve sexual and reproductive health [12]. The year 2012 marked its 40th anniversary [13]. While the quality of reproductive health in developing countries is clearly higher than in developing countries, major opportunity for health gains exist there also for women and future generations, particularly in economically disadvantaged or rural communities.

A growing understanding of periconception care

Exposure to teratogens and nutrient deficiency were linked to congenital defects during the last century, and these concepts dominated maternal–fetal research. In the 21st century, the greatest health gains stand to be made from research addressing more cryptic but pervasive ill-health outcomes with long latencies

that are functional rather than structural, which emerge through interactions between the individual and the environment, and which have effects that endure across generations.

There are multiple points of vulnerability throughout the pre-birth and post-birth phases of life that are prone to the positive or negative impact of internal and external influences. We and others have shown that the very earliest stages of embryogenesis are most susceptible. At this time, the organism is rapidly developing and must exhibit great plasticity to best survive the number and scale of critical transitions from zygote to fetus [14].

The earliest determinant of life potential is the oocyte, the developmental competence of which is influenced by the local hormonal, growth factor, and cellular environment of the ovarian follicle in which it grows [15, 16]. After fertilization, developmental plasticity is desirable so that the early embryo can respond to the demands and opportunities of the outside world by adaptation, rather than by adhering to a standard fixed phenotype that may be inappropriate to the changing external environment. Plasticity can be exerted at the cellular level by adjustment of cell numbers and fate, and at the molecular level by changes in gene expression pathways or the more permanent effects of epigenetics [17–19]. Together these processes exert modifications through which the periconception environment can modulate the phenotype to “best suit” the prevailing or predicted after-birth environment. Cytokines and growth factors secreted by maternal tract cells, along with metabolic substrates and other physiochemical agents, are implicated as signals through which the embryo senses its local environment [20]. The balance of pro-survival and pro-apoptotic cytokines can influence embryo survival and program epigenetic changes in response to environmental cues [21]. Remarkably, these cytokines are affected not only by the woman’s environment and her health but also by those of her partner. The male seminal fluid delivers signalling molecules which interact with female tissues to alter gene expression and impact the molecular composition of the oviduct and uterine fluids at conception [6, 22]. This seminal fluid priming can influence endometrial receptivity for implantation, the progression of pregnancy, and the health of offspring after birth [5, 22]. Health exposures in the male partner, for example a low protein or high fat diet, or exposure to endocrine disrupting chemicals, can change the composition of seminal fluid and interfere with its ability to promote immune tolerance in the female partner [23–25].

The reason the periconception phase of early development is so vulnerable may reflect the importance of this phase as an opportunity for evolutionary selection and adaptation to be exerted. From an evolutionary perspective, imposing constraints and selection pressures upon the conceptus is necessary to avoid unfavourable investment of reproductive resources and to maximize offspring health. The mammalian female has limited opportunities for pregnancy during her reproductive life span and each pregnancy costs resources and poses a risk to her own health. The majority of early embryos fail to survive and only ~60% of embryos that implant persist beyond the second week.

Decreased implantation rates result from the absence or suppression of molecules essential for endometrial receptivity, the mechanisms of which are diverse and include abnormal cytokine and hormonal signalling as well as epigenetic alterations [26, 27]. There are evolutionary advantages associated with active female-controlled processes for discerning the suitability of male gametes and embryos [28]. The female immune response is “aware” of fetal transplantation antigens and is competent to discriminate

the reproductive fitness and compatibility of the male partner and the integrity and developmental competence of the conceptus tissue [29, 30]. Since the immune response is modulated by the individual’s infectious, inflammatory, stress, nutritional, and metabolic status, immune influence on progression or disruption of pregnancy may be further influenced by environmental stressors and resource availability. Emerging evidence suggests that the immune system can integrate these signals to exert executive quality control to either accommodate or reject the conceptus. “Immune-mediated quality control” facilitates optimal female reproductive investment and explains the evolutionary advantage of engaging the immune system in the events of reproduction [21, 31].

With plasticity and maternal selection comes the risk of poor outcomes—when embryo sensing of the external environment fails to properly indicate and match the reality, where compromises made to favour immediate survival are suboptimal for longevity of life after birth, or when maternal quality control systems are inappropriately executed or otherwise faulty. In broad terms, it seems that extreme adaptation causes loss of functional capacity and resistance to future stressors, while maintenance of capacity in early intrauterine life improves the likelihood of subsequent health and resilience in adulthood [32]. If capacity is lost in early embryonic and fetal development, the possibility of dysfunction in later life becomes higher (Figure 34.1).

The permanent effects of exerting early plasticity are often not readily observable until later in fetal or postnatal life. Changes in cell numbers and lineage allocation or in gene or protein expression in blastocysts due to perturbation in the local physiochemical or cytokine environment [33–35] cause differences in placental structure and nutrient transport function, which is the key limiting factor in fetal growth [36, 37]. Disturbance to epigenetic regulation of both imprinted and non-imprinted genes, caused by various environmental factors, can lead to abnormal placental development and function with possible consequences for maternal morbidity, fetal development, and disease onset in later life [38]. This occurs because in adults, susceptibility or resilience to stressors and insults that precipitate disease are affected

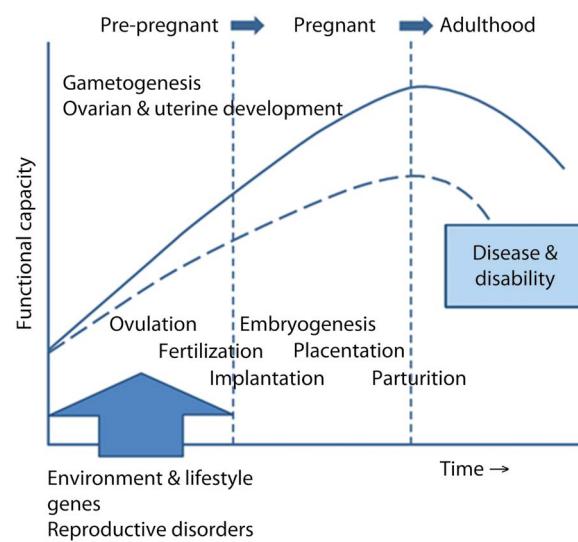


FIGURE 34.1 Adaptation to adverse influences in early life causes loss of functional capacity after birth.

by the cellular composition of tissues, particularly the numbers of stem and pluripotent cells and the epigenetic programming of gene regulation laid down at this time [39].

Experimental perturbations at various stages of pregnancy implicate the first days after conception as the most susceptible period for later fetal and postnatal growth impairment [40]. Altered embryo development, or insufficient maternal support of the conceptus at implantation can lead to later miscarriage, or “shallow” placental development resulting in preeclampsia, fetal growth restriction, and/or preterm delivery [41, 42]. In turn, these conditions affect growth after birth and impart a “thrifty” phenotype that leads to metabolic disorder and the onset of chronic disease. Thus, maternal stress in the periconception period due to nutritional, metabolic, immunological, infectious, pharmacological, or psychosocial perturbations can exert subtle but permanent alterations in the life-course trajectory of the offspring (Figure 34.2).

Maternal reproductive disorders such as polycystic ovary syndrome (PCOS), obesity, endometriosis, and ovulation disorders influence periconception events, alter endometrial receptivity and quality control sensing, and impart stress on the gametes and embryo (Figure 34.2) [43, 44]. These reproductive disorders share inflammatory pathways, hormonal aberrations, decidual senescence, and vascular abnormalities that may impair pregnancy success through common mechanisms [45]. Chronic sexually transmitted infection is another key factor that influences the maternal environment. Either in combination or alone, these disorders result in an increased risk of preterm birth, fetal growth restriction, placental pathologies, and hypertensive disorders. Systemic hormonal aberrations, and inflammatory and metabolic factors acting on the endometrium, myometrium, cervix, and placenta are all associated with an altered milieu during implantation and pregnancy, thus contributing to the genesis of obstetric complications [45].

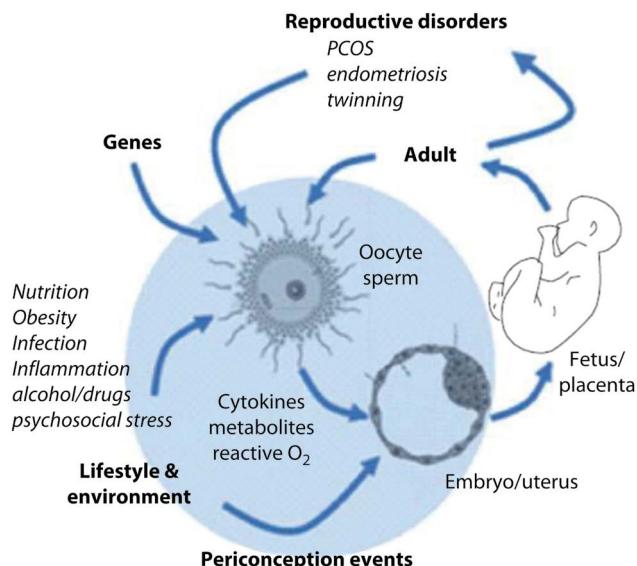


FIGURE 34.2 Periconception events are influenced by genes, a range of lifestyle/environmental factors, and maternal factors including reproductive disorders to impact fetal development and adult outcome.

ART, which is now the method of conception for many children in developed countries, also potentially inflicts substantial stress on the embryo [46]. We now recognize that *in vitro* embryo culture in media deficient in maternal signalling factors, the gonadotropin-induced altered hormone environment imposed on the oocyte prior to conception, and the disordered endometrium in a stimulated cycle, each predispose to growth restriction and attendant life-long effects on children [46–50]. Clinical practice until now shows that the *in vitro* culture of human embryos does not confer major adverse effects on the offspring but possible consequences in late childhood or adulthood are still to be determined, keeping in mind that even the first children conceived by ART are still relatively young [51]. There is evidence that transgenerational programming is a key factor in PCOS and that other forms of reproductive dysfunction can be programmed in utero [52–54]. Competition in the uterus through twinning or higher order multiple pregnancy, irrespective of ART or spontaneous occurrence, also causes fetal growth impairment and can bring adverse life-long consequences [55].

These and many other observations indicate the potential for optimizing fertility, pregnancy, and offspring health by planning prior to the onset of pregnancy and maximizing health particularly in early pregnancy. This has been classified as preconception care [56] with many publications advocating the potential benefits of individual and community participation in planning for women and men [57, 58].

Factors that affect fertility

Weight, exercise, and nutrition

The prevalence of overweight in young women and men of reproductive age is steadily increasing [59–61]. There is evidence to show that female weight disorders, both under- and overweight, impair spontaneous fertility [62, 63]. Obesity has been linked to male fertility because of its effects on reproductive physiology, alterations to hormone production, and adverse effects on sperm genetic integrity [64]. Both paternal and maternal obesity can negatively affect ART outcomes [65, 66]. Female obesity has been shown to be associated with poor pregnancy outcomes, including increased rates of congenital abnormalities, caesarean delivery, pre-eclampsia, gestational diabetes, fetal macrosomia, stillbirth, and post-term pregnancy [67, 68]. It has been reported that physical activity improves cardiovascular risk factors, hormonal profile, and reproductive function. These improvements include a decrease in abdominal fat, blood glucose, blood lipids, and insulin resistance [69], as well as improvements in menstrual cyclicity and ovulation [70], increased rates of clinical pregnancy and live births [71], decreases in testosterone levels and Free Androgen Index (FAI), and increases in sex hormone binding globulin (SHBG) [72]. Existing evidence from large randomized controlled trials prior to fertility intervention shows no improvement in live birth or other fertility outcomes after weight loss interventions in women with obesity and infertility [73–75]. It remains controversial to recommend weight loss for women with obesity prior to infertility treatment, including ART [76]. There is substantial evidence pointing to the adverse effect of obesity at the level of the egg and embryo [77, 78]. Recent data also suggests a non-genomic transfer of metabolic disorders via sperm, and, if confirmed, this implies much more attention needs to be paid to optimization of male and female health and nutrition prior to pregnancy [79, 80].

Diet

There are a number of dietary factors that have been investigated in regards to reproduction:

- *Vitamins.* In a 2018 review, antioxidant supplements and supplemental vitamin D were considered unlikely to have an impact on reproductive health outcomes, and there are limited studies addressing this [81]. However, given the high proportion of vitamin D deficiency across many groups, particularly in women with dark skin or little sun exposure, screening for vitamin D deficiency prior to pregnancy is recommended, along with a vitamin D supplement, where necessary [82]. While there is increasing, positive evidence for an association between folic acid supplementation and fecundability [83], further studies in this area are warranted. A daily 400- μ g folic acid supplement in the one month prior to pregnancy for women is recommended by the WHO, and where there is a higher risk of abnormality, a supplemental intake of 5 mg is recommended [84]. This is not specifically to improve chances of pregnancy but to reduce the risk of congenital malformations. In terms of vitamin A, it is recommended that women avoid the retinol form of vitamin A and foods containing this form of vitamin A [82, 85, 86].
- *Iodine.* Many women seeking pregnancy are iodine deficient, thus iodine is often added to prenatal supplements or foods [87]. All women who are pregnant, and breastfeeding, are advised to take an iodine supplement of 250 μ g each day, and in situations where it is difficult to reach pregnant women, a 150- μ g supplement to all women of reproductive age is advised by the WHO [88].
- *Male antioxidants.* Oxidative stress is frequently described in infertile males [89]. A recent Cochrane review shows antioxidant supplementation in sub-fertile males may improve live birth rates, but the overall quality of existing evidence remains low and therefore further trials are needed to confirm the findings [90]. While several commercial supplemental preparations exist, dietary patterns favouring the consumption of seafood, poultry, nuts, whole grains, fruits, and vegetables should be the first step to support fertility [91].
- *Alcohol.* Existing evidence suggests an inverse association between alcohol consumption and fecundability, especially moderate to heavy drinking during the luteal phase, and heavy drinking in the ovulatory window [92]. Nevertheless, there is clear evidence and strong biological plausibility for adverse reproductive effects. There are clear adverse reproductive effects for women, including decreased ovary volume and lower number of eggs containing follicles [93]; and for men, testicular shrinkage and decreased testosterone concentrations and sperm counts [94]. The role of alcohol in the fetal alcohol syndrome is well-known [95], thus limiting or consuming no alcohol is the safest option while planning a pregnancy.
- *Caffeine.* Caffeine is the most popular neurostimulant and is found in drinks and foods across all cultures. A high consumption of caffeine may be associated with impaired fecundity, although the evidence is not conclusive [82, 96–98]. While a safe level of caffeine has not been defined, it is recommended to keep this below 200–300 mg per day (less than two cups of coffee per day) [99–101].

• *Individual foods.* Reducing trans fatty acids, saturated fatty acids, and discretionary food intake (fast food and sugar-sweetened beverages) appear associated with improvements in live birth, clinical pregnancy rates, and related ART outcomes [102]. A recent review on male diet and sperm function also indicated that higher intake of fruits and vegetables was associated with increased sperm count and motility, whereas a higher intake of fat-rich foods and sweets may decrease sperm quality [103, 104].

• *Dietary patterns.* While many studies have focused on single nutrients or foods in relation to fertility, there is a continuing shift in nutritional epidemiology from individual nutrients and foods to dietary patterns and the overall diet, because we eat foods and not nutrients. Most, but not all, studies demonstrate a relationship between higher adherence to a Mediterranean diet, which is high in omega-3 fatty acids, some antioxidants and vitamins, and low in saturated- and trans fatty acids [105], or a “pro-fertility” diet, characterized by a higher intake of low-pesticide fruits and vegetables, whole grains, seafood, dairy, and soy foods, and clinical pregnancy rates or live birth [81, 106–109]. In men, the Mediterranean diet also has been positively associated with semen parameters in some [110] but not all studies [111, 112]. A recent study also reported no association with other a priori dietary patterns, such as the Dietary Approaches to Stop Hypertension diet, or American Heart Association diet recommendations [112].

Smoking

Smoking can affect all stages of reproduction, including folliculogenesis, steroidogenesis, embryo transport, endometrial receptivity, endometrial angiogenesis, uterine blood flow, and uterine myometrium [113]. However, the effect of smoking on fertility is underestimated by the public [114]. A 2019 meta-analysis demonstrated smoking to have a significant impact on the quantity and quality of sperm in infertile male participants, including lower sperm count and an increase in the number of morphological defects [115, 116]. For female smokers, a range of adverse outcomes were detected compared to non-smokers, including decreases in live birth and clinical pregnancy rate per cycle, a decrease in the number of retrieved oocytes, average fertilization rate, as well as a significantly increased miscarriage rate per pregnancy [115, 116]. Sperm studies have shown increased oxidative stress, a lower sperm count, and abnormal sperm fertilizing capacity, with a significantly reduced chance of pregnancy in a female partner [117, 118]. Passive smoking is a contributing factor in increasing complications in pregnancy as well as in IVF cycles [119, 120]. There are many studies showing that intervention programs for smoking can be successful.

Recreational drugs

Recreational drugs are those used without medicinal need, and can include cocaine, cannabis (marijuana), or methamphetamine. Of these, cannabis is by far the most widely used in women of reproductive age [121], and with high levels reported in men [122]. Cannabis use in women with a history of pregnancy loss during the pre-conceptual period was associated with reduced fecundability [123]. Cannabis use during pregnancy has adverse effects on the fetus, including a high frequency of severe neonatal morbidity and death [124], and possibly intellectual disability and learning disorders post birth [125]. In men there is sufficient evidence demonstrating that cannabis use decreases sperm

concentration, motility, and morphology, making it more difficult to conceive [126], with significant effects on the genetic makeup of sperm after cannabis intake, which can be inherited by the fetus [127]. Cocaine impairs ovarian responsiveness and alters sperm function [128, 129], whereas heroin and methadone also have significant effects [62, 130]. Anabolic steroids can reduce testicular sperm production, while the role of other lifestyle drugs is still to be explored [131].

Other prescription drugs

There are many drugs that appear to affect fertility, congenital abnormalities, and alter reproductive outcomes [82, 132]. These should be assessed during initial consultations, and the patient should be recommended to seek alternatives if actively trying to become pregnant.

Stress

There is growing evidence that psychosocial stress is associated with negative reproductive outcomes, including pregnancy rates [133]. Stress triggers the activation of the hypothalamic-pituitary-adrenal axis and the sympathetic-adrenal-medullary axis [134]. The hormones secreted by these systems after stressful stimuli result in an abnormal, prolonged, and/or excessive stress-induced body's set-up that can potentially produce long-term neuroendocrine changes, affecting fertility [135, 136]. Appropriate counselling and lifestyle adjustments may ameliorate these effects [133]. Based on the best available evidence in the literature, the European Society of Human Reproduction and Embryology (ESHRE) has recently developed guidelines for routine psychosocial care at infertility and medically assisted reproduction (MAR) clinics [137].

Environmental pollutants

There is considerable interest regarding environmental toxins and pollutants and the effects on reproductive health. Collectively, these environmental pollutants are often referred to as endocrine disrupting chemicals (EDCs)—chemicals which can interfere with normal reproductive systems or hormones. EDCs can enter the environment and food chain through different processes, including emissions, during manufacture or processing, or leaching from products, to become available for human uptake [138]. A 2019 review that investigated a range of EDCs on the ability to become pregnant showed mostly weak associations between individual EDCs and conception rates; for example, phthalate exposure in women is associated with both a longer and shorter time to pregnancy, and there is also some evidence for men with a longer time to pregnancy due to reduced sperm quality [139, 140]. Higher levels of bisphenol A were also shown in women experiencing infertility [141]. Until further evidence becomes available, a precautionary approach is recommended, including the limiting of EDC exposure through good hygiene practices, washing fruits and vegetables, avoiding needless exposure to outdoor and indoor chemicals, and minimizing the use of personal care and cosmetic products.

Vaccinations

There is little data on the impact of vaccinations on fertility, but the serious consequences of becoming infected with rubella, herpes zoster, varicella zoster, and influenza indicate that immunization prior to pregnancy is appropriate [85, 142]. Although long-term data on Covid-19 vaccination is lacking, emerging evidence on short-term data suggests mRNA vaccines do not result in fertility problems in women or men [143].

Sexually transmitted diseases

It is increasingly evident that bacterial and viral infections of the reproductive tissues can alter immune and inflammatory parameters in such a way as to impede periconception events and reduce fertility. The recommendation is that couples (both partners) should seek advice from their clinical care provider regarding detection and treatment of any infection of the reproductive tract, remembering that many (such as chlamydia) are widespread in the community and may not necessarily result in signs or symptoms. The role of the vaginal and endometrial microbiome is receiving increasing attention [144, 145].

Occupational factors

Evidence suggests that the circadian clock regulates each part of the reproductive axis from timing of neuronal activity in hypothalamic neurons to the day-night variation in the release of pregnancy hormones [115, 116]. Dysregulation of circadian rhythms, as often occurs with shift work and jet lag, contributes to altered menstrual cycles [115, 116], changes in follicular stage length [115, 116], and FSH concentrations [115, 116]. Other common workplace exposures, such as prolonged working hours, lifting, standing and heavy physical workload, may also increase the risk of adverse obstetric and neonatal outcomes [146].

Pre-pregnancy preparation

Given the theoretical and practical background to periconception health, the desire of infertile couples to seek specialist treatment and the opportunity to favourably influence outcomes of fertility treatment, all clinics should have a program to assess adverse maternal and paternal genetic and lifestyle influences on reproduction, and an intervention protocol to minimize their detrimental effects. This is best achieved at the couple's first appointment with the clinic doctor or nurse. A comprehensive interview covering past medical and family history, medications and environmental exposures, diet, risky behaviours (including pharmaceutical and recreational drugs, smoking, and alcohol), exercise, and vaccinations might be followed by an appropriate examination. Action can then be advised while there is time for an effective plan to be instituted by the clinic and couple (Figure 34.3). This may be as simple as taking a folic acid supplement and changing diet to optimize the periconception environment, through to active weight loss programs, smoking cessation interventions, and elimination of inappropriate alcohol and drug use. In the past decade, several systematic reviews have examined preconception care interventions and reported improvements in maternal and child outcomes in some but not all studies [115, 116]. A 2017 scoping review on preconception interventions highlighted that while progress has been made in intervening on preconception health, further work is needed in terms of designing interventions for partners/men, and how best to deliver preconception care [115, 116]. Several groups have described programs for weight loss in the context of a fertility clinic with the best known being that by Clark from Adelaide (Fertility Fitness), Australia [147, 148] as well as the FAST study [149]. In this program, 5% weight loss was associated with a dramatic improvement in spontaneous and IVF pregnancy rates. Dokras and colleagues have published compelling evidence for significant weight loss in a PCOS population that could be applied to other groups [150]. Other popular community or expert-based facilities are available in the general community to improve lifestyle prior to pregnancy or while actively intervening.

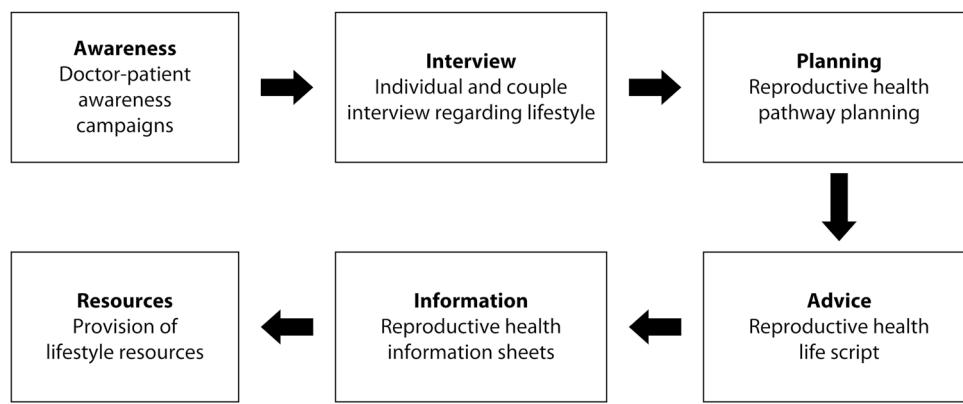


FIGURE 34.3 An approach to assessing and managing lifestyle in a clinical setting.

Governments and healthcare systems have a responsibility to facilitate and encourage various aspects of preconception care, including promoting vaccination, controlling alcohol and smoking use, providing a safe workplace, and giving general reproductive education (Figure 34.4). The clinic and individual, however, have an even greater role in safeguarding reproductive security by ensuring any pregnancy is conceived with gametes and embryos that have had the best chance to achieve their full genetic potential.

Summary

In summary, there is compelling evidence that external and endogenous events in women and men impact preconception and very early pregnancy to benefit or hinder the later health of the neonate, child, and adult. Events in the pre- and peri-implantation period, spanning gametogenesis, conception, and early placental morphogenesis, have the power to impart long-term susceptibility or resilience to later health challenges in our children and community.

Defining the nature and actions of these external and endogenous events is now attainable. We know several of the key

interlocutory signals between the oocyte and follicle, the sperm and oocyte, and the conceptus and uterus, but their full identity and interaction with environmental factors, reproductive disorders, and genetic background remains to be elucidated. Some of the most potent stressors for embryos and gametes are lifestyle factors—very young or advanced age, obesity, sexually transmitted infection, drugs, alcohol, diet, vitamin deficiency, and psychosocial stress. Further understanding on how these factors affect periconception biology will contribute to public health initiatives aimed at modifying behaviours and through educating teenagers and prospective parents. Similarly, the maternal reproductive disorders that impact early development are amenable to improved diagnosis and clinical treatments. Defining such effects on the ovary and uterus, gametes, embryo, and placenta, and their interactions with environmental factors in the context of different genetic settings, is essential to focus and prioritize clinical interventions. Despite the complexity in these interactions, there is evidence that several stressors converge through critical common inflammatory and metabolic pathways. Therefore, the prospect of identifying interventions or drug targets to minimize, reverse, or protect against adverse early environments is likely to be achievable.



FIGURE 34.4 A society-wide approach to achieving lifestyle changes.

References

1. World Health Organization. *WHO. World Health Statistics 2019. Monitoring health for the SDGs. Sustainable Development Goals.* Available at: <https://apps.who.int/iris/bitstream/handle/10665/324835/9789241565707-eng.pdf>.
2. Lane M, Robker RL, Robertson SA. Parenting from before conception. *Science.* 2014;345(6198):756–60.
3. Steegers-Theunissen RP, et al. The periconceptional period, reproduction and long-term health of offspring: The importance of one-carbon metabolism. *Hum Reprod Update.* 2013;19(6):640–55.
4. Norman RJ. 2015 RANZCOG Arthur Wilson Memorial Oration ‘From little things, big things grow: The importance of periconception medicine’. *Aust N Z J Obstet Gynaecol.* 2015;55(6):535–40.
5. Robertson SA, Care AS, Moldenhauer LM. Regulatory t cells in embryo implantation and the immune response to pregnancy. *J Clin Invest.* 2018;128(10):4224–35.
6. Robertson SA, Sharkey DJ. Seminal fluid and fertility in women. *Fertil Steril.* 2016;106(3):511–9.
7. Barker DJ, Clark PM. Fetal undernutrition and disease in later life. *Rev Reprod.* 1997;2(2):105–12.
8. United Nations International Conference on Population and Development (ICPD). Programme of Action of the United Nations International Conference on Population & Development. Egypt, Cairo: Reproductive Rights and Reproductive Health, 1994.
9. Mongella G. The United Nations: Report of the Fourth World Conference on Women. Beijing, China, 1996.
10. Sachs JD. Investing in Development: A Practical Plan to Achieve the Millennium Development Goals. London: Earthscan, 2005.
11. Sachs JD. Investing in Development: A Practical Plan to Achieve the Millennium Development Goals. 2005.
12. WHO. Research on sexual and reproductive health throughout the world. 2015. Geneva (CH): WHO; 2015.
13. Benagiano G, et al. The special programme of research in human reproduction: Forty years of activities to achieve reproductive health for all. *Gynecol Obstet Invest.* 2012;74(3):190–217.
14. Thompson JG, Lane M, Robertson SA. Adaptive responses of embryos to their microenvironment and consequences for post-implantation development. In: Early Life Origins of Health and Disease. Owens JS, Wintour M (eds). New York: Landes Bioscience, pp. 58–69, 2005.
15. Sutton ML, Gilchrist RB, Thompson JG. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update.* 2003;9(1):35–48.
16. Mtango NR, Potireddy S, Latham KE. Oocyte quality and maternal control of development. *Int Rev Cell Mol Biol.* 2008;268:223–90.
17. Young LE. Imprinting of genes and the barker hypothesis. *Twin Res.* 2001;4(5):307–17.
18. Morgan HD, et al. Epigenetic reprogramming in mammals. *Hum Mol Genet.* 2005;14(1):R47–58.
19. Kaminsky ZA, et al. DNA methylation profiles in monozygotic and dizygotic twins. *Nat Genet.* 2009;41(2):240–5.
20. Robertson SA, et al. Peri-conceptual cytokines—setting the trajectory for embryo implantation, pregnancy and beyond. *Am J Reprod Immunol.* 2011;66(Suppl 1):2–10.
21. Robertson SA, et al. Female tract cytokines and developmental programming in embryos. *Adv Exp Med Biol.* 2015;843:173–213.
22. Bromfield JJ, et al. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc Natl Acad Sci U S A.* 2014;111(6):2200–5.
23. Schjenken JE, et al. High-fat diet alters male seminal plasma composition to impair female immune adaptation for pregnancy in mice. *Endocrinology.* 2021;162(10):bqab123.
24. Skerrett-Byrne DA, et al. Proteomic dissection of the impact of environmental exposures on mouse seminal vesicle function. *Mol Cell Proteomics.* 2021;20:100107.
25. Watkins AJ, et al. Paternal diet programs offspring health through sperm- and seminal plasma-specific pathways in mice. *Proc Natl Acad Sci U S A.* 2018;115(40):10064–9.
26. Robertson SA, et al. Embryotoxic cytokines—potential roles in embryo loss and fetal programming. *J Reprod Immunol.* 2018;125:80–88.
27. Robertson SA, Moldenhauer LM. Immunological determinants of implantation success. *Int J Dev Biol.* 2014;58(2–4):205–17.
28. Eberhard WG. Postcopulatory sexual selection: Darwin’s omission and its consequences. *Proc Natl Acad Sci U S A.* 2009;106(Suppl 1):10025–32.
29. Robertson SA, et al. Activating t regulatory cells for tolerance in early pregnancy - the contribution of seminal fluid. *J Reprod Immunol.* 2009;83(1–2):109–16.
30. Trowsdale J, Betz AG. Mother’s little helpers: Mechanisms of maternal-fetal tolerance. *Nat Immunol.* 2006;7(3):241–6.
31. Robertson SA. Immune regulation of conception and embryo implantation—all about quality control? *J Reprod Immunol.* 2010;85(1):51–7.
32. Watkins AJ, Papenbrock T, Fleming TP. The preimplantation embryo: Handle with care. *Semin Reprod Med.* 2008;26(2):175–85.
33. Lane M, Gardner DK. Differential regulation of mouse embryo development and viability by amino acids. *J Reprod Fertil.* 1997;109(1):153–64.
34. Kwong WY, et al. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development.* 2000;127(19):4195–202.
35. Sjöblom C, et al. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology.* 2005;146(5):2142–53.
36. Tam PP. Postimplantation development of mitomycin C-treated mouse blastocysts. *Teratology.* 1988;37(3):205–12.
37. Godfrey KM. The role of the placenta in fetal programming—a review. *Placenta.* 2002;23(Suppl A):S20–7.
38. Nelissen EC, et al. Epigenetics and the placenta. *Hum Reprod Update.* 2011;17(3):397–417.
39. Vaiserman AM. Epigenetic programming by early-life stress: Evidence from human populations. *Dev Dyn.* 2015;244(3):254–65.
40. Hoet JJ, Ozanne S, Reusens B. Influences of pre- and postnatal nutritional exposures on vascular/endocrine systems in animals. *Environ Health Perspect.* 2000;108(Suppl 3):563–8.
41. Fowden AL, et al. The placenta and intrauterine programming. *J Neuroendocrinol.* 2008;20(4):439–50.
42. Maltepe E, Bakardjieva AI, Fisher SJ. The placenta: Transcriptional, epigenetic, and physiological integration during development. *J Clin Invest.* 2010;120(4):1016–25.
43. Davies MJ, Norman RJ. Programming and reproductive functioning. *Trends Endocrinol Metab.* 2002;13(9):386–92.
44. Lord JM, Norman R. Obesity, polycystic ovary syndrome, infertility treatment: Lifestyle modification is paramount. *BMJ.* 2006;332(7541):609.
45. Vannuccini S, et al. Infertility and reproductive disorders: Impact of hormonal and inflammatory mechanisms on pregnancy outcome. *Hum Reprod Update.* 2016;22(1):104–15.
46. Thompson JG, et al. Epigenetic risks related to assisted reproductive technologies: Short- and long-term consequences for the health of children conceived through assisted reproduction technology: More reason for caution? *Hum Reprod.* 2002;17(11):2783–6.
47. De Rycke M, Liebaers I, Van Steirteghem A. Epigenetic Risks related to assisted reproductive technologies: Risk analysis and epigenetic inheritance. *Hum Reprod.* 2002;17(10):2487–94.
48. Basatemur E, Sutcliffe A. Follow-up of children born after ART. *Placenta.* 2008;29(Suppl B):135–40.
49. Richani D, et al. Effect of epidermal growth factor-like peptides on the metabolism of in vitro- matured mouse oocytes and cumulus cells. *Biol Reprod.* 2014;90(3):49.

50. Sharkey AM, Macklon NS. The science of implantation emerges blinking into the light. *Reprod Biomed Online.* 2013;27(5):453–60.
51. Chronopoulou E, Harper JC. IVF culture media: Past, present and future. *Hum Reprod Update.* 2015;21(1):39–55.
52. Dumesic DA, Abbott DH, Padmanabhan V. Polycystic ovary syndrome and its developmental origins. *Rev Endocr Metab Disord.* 2007;8(2):127–41.
53. Norman RJ, Hickey T, Moran L. Genetic/epigenetic and environmental origins of PCOS. *Early Hum Dev.* 2007;83:S43–4.
54. Rhind SM, Rae MT, Brooks AN. Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction.* 2001;122(2):205–14.
55. Davies MJ. Fetal programming: The perspective of single and twin pregnancies. *Reprod Fertil Dev.* 2005;17(3):379–86.
56. Johnson K, Posner SF, Biermann J, Cordero JE, Atrash HK, Parker CS, Boulet S, Curtis MG. Recommendations to Improve Preconception Health and Health Care—United States. A Report of the CDC/ATSDR Preconception Care Work Group and the Select Panel on Preconception Care. April 21, 2006;55(RR06);1–23. Available from: <https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5506a1.htm>.
57. Daly M, Kipping RR, Tinner LE, Sanders J, JW White. Preconception exposures and adverse pregnancy, birth and postpartum outcomes: Umbrella review of systematic reviews. *Paediatr Perinat Epidemiol.* 2021;36(2):288–99.
58. Stephenson J, et al. Before The beginning: Nutrition and lifestyle in The preconception period and its importance for future health. *Lancet.* 2018;391(10132):1830–41.
59. Dutton GR, et al. 25-year weight gain in a racially balanced sample of U.S. Adults: The CARDIA study. *Obesity (Silver Spring).* 2016;24(9):1962–8.
60. Collaboration NCDRF. Trends in adult body-mass index in 200 countries from 1975 to 2014: A pooled analysis of 1698 population-based measurement studies with 19.2 million participants. *Lancet.* 2016;387(10026):1377–96.
61. Gomersall SR, Dobson AJ, Brown WJ. Weight gain, overweight, and obesity: Determinants and health outcomes from the Australian longitudinal study on Women's health. *Curr Obes Rep.* 2014;3(1):46–53.
62. Ramlau-Hansen CH, et al. Subfecundity in overweight and obese couples. *Hum Reprod.* 2007;22(6):1634–7.
63. Hassan MA, Killick SR. Negative lifestyle is associated with a significant reduction in fecundity. *Fertil Steril.* 2004;81(2):384–92.
64. Shukla KK, et al. Recent scenario of obesity and male fertility. *Andrology.* 2014;2(6):809–18.
65. Campbell JM, et al. Paternal obesity negatively affects male fertility and assisted reproduction outcomes: A systematic review and meta-analysis. *Reprod Biomed Online.* 2015;31(5):593–604.
66. Rittenberg V, et al. Effect of body mass index on IVF treatment outcome: An updated systematic review and meta-Analysis. *Reprod Biomed Online.* 2011;23(4):421–39.
67. Broughton DE, Moley KH. Obesity and female infertility: Potential mediators of obesity's impact. *Fertil Steril.* 2017;107(4):840–47.
68. Mission JF, Marshall NE, Caughey AB. Obesity in pregnancy: A big problem and getting bigger. *Obstet Gynecol Surv.* 2013;68(5):389–99.
69. Lavie CJ, et al. Sedentary behavior, exercise, and cardiovascular health. *Circ Res.* 2019;124(5):799–815.
70. Hakimi O, Cameron LC. Effect of exercise on ovulation: A systematic review. *Sports Med.* 2017;47(8):1555–67.
71. Rao M, Zeng Z, Tang L. Maternal physical activity before IVF/ICSI cycles improves clinical pregnancy rate and live birth rate: A systematic review and meta-analysis. *Reprod Biol Endocrinol.* 2018;16(1):11.
72. Orio F, et al. Effects of physical exercise on the female reproductive system. *Minerva Endocrinol.* 2013;38(3):305–19.
73. Einarsson S, et al. Weight reduction intervention for obese infertile women prior to IVF: A randomized controlled trial. *Hum Reprod.* 2017;32(8):1621–30.
74. Legro RS, et al. Effects of preconception lifestyle intervention in infertile women with obesity: The FIT-PLESE randomized controlled trial. *PLoS Med.* 2022;19(1):e1003883.
75. Mutsaerts MA, et al. Randomized trial of a lifestyle program in obese infertile women. *N Engl J Med.* 2016;374(20):1942–53.
76. Legro RS. Mr. Fertility authority, tear down that weight wall! *Hum Reprod.* 2016;31(12):2662–64.
77. Wu LL, Norman RJ, Robker RL. The impact of obesity on oocytes: Evidence for lipotoxicity mechanisms. *Reprod Fertil Dev.* 2011;24(1):29–34.
78. McPherson NO, et al. When two obese parents are worse than one! Impacts on embryo and fetal development. *Am J Physiol Endocrinol Metab.* 2015;309(6):E568–81.
79. Fullston T, et al. Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *FASEB J.* 2013;27(10):4226–43.
80. Ng SF, et al. Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring. *Nature.* 2010;467(7318):963–6.
81. Gaskins AJ, Chavarro JE. Diet and fertility: A review. *Am J Obstet Gynecol.* 2018;218(4):379–89.
82. Anderson K, Nisenblat V, Norman R. Lifestyle factors in people seeking infertility treatment – A review. *Aust N Z J Obstet Gynaecol.* 2010;50(1):8–20.
83. Schaefer E, Nock D. The impact of preconceptual multiple-micronutrient supplementation on female fertility. *Clin Med Insights Womens Health.* 2019;12:1179562X19843868.
84. World Health Organization. 2019. Periconceptional folic acid supplementation to prevent neural tube defects. e-Library of Evidence for Nutrition Actions (eLENA). Available at: <https://www.who.int/tools/elena/review-summaries/folate-periconceptional--effects-and-safety-of-periconceptional-oral-folate-supplementation-for-preventing-birth-defects>.
85. The Royal Australian College of General Practitioners RACGP House. Guidelines for Preventive Activities in General Practice, 8th edition. East Melbourne: Royal Australian College of General Practitioners, 2012.
86. SA Maternal & Neonatal Clinical Network. Vitamin and mineral supplementation in pregnancy, G.o.S.A. Department of Health, Editor, 2014.
87. Li M, Eastman CJ. The changing epidemiology of iodine deficiency. *Nat Rev Endocrinol.* 2012;8(7):434–40.
88. World Health Organization. WHO. e-Library of Evidence for Nutrition Actions (eLENA). Iodine supplementation in pregnant and lactating women. Available at: <https://www.who.int/tools/elena/bbc/iodine-pregnancy>
89. De Luca MN, et al. Oxidative stress and male fertility: Role of antioxidants and inositol. *Antioxidants (Basel).* 2021;10(8):1283.
90. Smits RM, et al. Antioxidants for male subfertility. *Cochrane Database Syst Rev.* 2019;3:CD007411.
91. Nassan FL, Chavarro JE, Tanrikut C. Diet and men's fertility: Does diet affect sperm quality? *Fertil Steril.* 2018;110(4):570–77.
92. Anwar MY, Marcus M, Taylor KC. The association between alcohol intake and fecundability during menstrual cycle phases. *Hum Reprod.* 2021;36(9):2538–48.
93. Van Heertum K, Rossi B. Alcohol and fertility: How much is too much? *Fertil Res Pract.* 2017;3:10.
94. Condorelli RA, et al. Chronic consumption of alcohol and sperm parameters: Our experience and the main evidences. *Andrologia.* 2015;47(4):368–79.
95. Mukherjee RA, et al. Low level alcohol consumption and the fetus. *BMJ.* 2005;330(7488):375–6.
96. Greenwood DC, et al. Caffeine intake during pregnancy, late miscarriage and stillbirth. *Eur J Epidemiol.* 2010;25(4):275–80.
97. Savitz DA, et al. Caffeine and miscarriage risk. *Epidemiology.* 2008;19(1):55–62.

98. Golding J. Reproduction and caffeine consumption—a literature review. *Early Hum Dev.* 1995;43(1):1–14.
99. Higdon JV, Frei B. Coffee and health: A review of recent human research. *Crit Rev Food Sci Nutr.* 2006;46(2):101–23.
100. Signorello LB, McLaughlin JK. Maternal caffeine consumption and spontaneous abortion: A review of the epidemiologic evidence. *Epidemiology.* 2004;15(2):229–39.
101. Nisenblat V NR. The effects of caffeine on reproductive outcomes in women. UpToDate, 2015.
102. Alesi S, et al. Assessing the influence of preconception diet on female fertility: a systematic scoping review of observational studies. *Hum Reprod Update.* 2023. <https://doi.org/10.1093/humupd/dmad018>.
103. Giahi L, et al. Nutritional modifications in male infertility: A systematic review covering 2 decades. *Nutr Rev.* 2016;74(2):118–30.
104. Salas-Huetos A, et al. The effect of nutrients and dietary supplements on sperm quality parameters: A systematic review and meta-analysis of randomized clinical trials. *Adv Nutr.* 2018;9(6):833–48.
105. Winter HG, et al. Can dietary patterns impact fertility outcomes? A systematic review and meta-analysis. *Nutrients.* 2023 May 31; 15(11):2589. doi: [10.3390/nu15112589](https://doi.org/10.3390/nu15112589).
106. Chavarro JE, et al. Diet and lifestyle in the prevention of ovulatory disorder infertility. *Obstet Gynecol.* 2007;110(5):1050–8.
107. Gaskins AJ, et al. Dietary patterns and outcomes of assisted reproduction. *Am J Obstet Gynecol.* 2019;220(6):567.e1–e18.
108. Karayiannis D, et al. Adherence to the mediterranean diet and IVF success rate among non-obese women attempting fertility. *Hum Reprod.* 2018;33(3):494–502.
109. Sanderman EA, Willis SK, Wise LA. Female dietary patterns and outcomes of in vitro fertilization (IVF): A systematic literature review. *Nutr J.* 2022;21(1):5.
110. Salas-Huetos A, Bullo M, Salas-Salvado J. Dietary patterns, foods and nutrients in male fertility parameters and fecundability: A systematic review of observational studies. *Hum Reprod Update.* 2017;23(4):371–389.
111. Mitsunami M, et al. A dietary score representing the overall relation of men's diet with semen quality in relation to outcomes of infertility treatment with assisted reproduction. *F S Rep.* 2021;2(4):396–404.
112. Salas-Huetos A, et al. Paternal adherence to healthy dietary patterns in relation to sperm parameters and outcomes of assisted reproductive technologies. *Fertil Steril.* 2022;117(2):298–312.
113. Dechanet C, et al. Effects of cigarette smoking on reproduction. *Hum Reprod Update.* 2011;17(1):76–95.
114. Practice Committee of the American Society for Reproductive Medicine. Smoking and infertility: A committee opinion. *Fertil Steril.* 2012;98(6):1400–6.
115. Hernandez TL, Mande A, Barbour LA. Nutrition therapy within and beyond gestational diabetes. *Diabetes Res Clin Pract.* 2018;145:39–50.
116. Wan CS, et al. Ethnic differences in dietary management of gestational diabetes mellitus: A mixed methods study comparing ethnic Chinese immigrants and Australian women. *J Acad Nutr Diet.* 2020;120(1):86–102.
117. Frey KA, et al. The clinical content of Preconception care: Preconception care for men. *Am J Obstet Gynecol.* 2008;199 (6 Suppl 2):S389–95.
118. Zitzmann M, et al. Male smokers have a decreased success rate for in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril.* 2003;79(Suppl 3):1550–4.
119. Pineles BL, Park E, Samet JM. Systematic review and meta-analysis of miscarriage and maternal exposure to tobacco smoke during pregnancy. *Am J Epidemiol.* 2014;179(7):807–23.
120. Neal MS, et al. Sidestream smoking is equally as damaging as mainstream smoking on IVF outcomes. *Hum Reprod.* 2005;20(9):2531–5.
121. Corsi DJ, Murphy MSQ, Cook J. The effects of Cannabis on female reproductive health across the life course. *Cannabis Cannabinoid Res.* 2021;6(4):275–87.
122. Cuttler C, Mischley LK, Sexton M. Sex differences in Cannabis use and effects: A cross-sectional survey of Cannabis users. *Cannabis Cannabinoid Res.* 2016;1(1):166–75.
123. Mumford SL, et al. Cannabis use while trying to conceive: A prospective cohort study evaluating associations with fecundability, live birth and pregnancy loss. *Hum Reprod.* 2021;36(5):1405–15.
124. Grzeskowiak LE, et al. The deleterious effects of cannabis during pregnancy on neonatal outcomes. *Med J Aust.* 2020;212(11):519–24.
125. Corsi DJ, et al. Maternal cannabis use in pregnancy and child neurodevelopmental outcomes. *Nat Med.* 2020;26(10):1536–40.
126. Payne KS, et al. Cannabis and male fertility: A systematic review. *J Urol.* 2019;202(4):674–81.
127. Schrott R, Murphy SK. Cannabis use and the sperm epigenome: A budding concern? *Environ Epigenet.* 2020;6(1):dvaa002.
128. Thyer AC, et al. Cocaine impairs ovarian response to exogenous gonadotropins in nonhuman primates. *J Soc Gynecol Investig.* 2001;8(6):358–62.
129. George VK, et al. Effects of long-term cocaine exposure on spermatogenesis and fertility in peripubertal male rats. *J Urol.* 1996;155(1):327–31.
130. Ragni G, et al. Gonadal function in male heroin and methadone addicts. *Int J Androl.* 1988;11(2):93–100.
131. Pasqualotto FF, et al. Effects of medical therapy, alcohol, smoking, and endocrine disruptors on male infertility. *Rev Hosp Clin Fac Med Sao Paulo.* 2004;59(6):375–82.
132. Pandiyan N. Medical drugs impairing fertility. In: *Reproductive Health and the Environment.* Nicolopoulou-Stamati P, Hens L, Howard CV, (eds.). Dordrecht, The Netherlands: Springer, pp. 187–205, 2007.
133. Rooney KL, Domar AD. The relationship between stress and infertility. *Dialogues Clin Neurosci.* 2018;20(1):41–47.
134. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci.* 2009;10(6):397–409.
135. Kaufman J, et al. Effects of early adverse experiences on brain structure and function: Clinical implications. *Biol Psychiatry.* 2000;48(8):778–90.
136. Negro-Vilar A. Stress and other environmental factors affecting fertility in men and women: Overview. *Environ Health Perspect.* 1993;101(Suppl 2):59–64.
137. Gameiro S, et al. ESHRE guideline: Routine psychosocial care in infertility and medically assisted reproduction—a guide for fertility staffdagger. *Hum Reprod.* 2015;30(11):2476–85.
138. Gore AC, et al. EDC-2: The endocrine Society's second scientific statement on endocrine-disrupting chemicals. *Endocr Rev.* 2015;36(6):E1–E150.
139. Di Nisio A, Foresta C. Water and soil pollution as determinant of water and food quality/contamination and its impact on male fertility. *Reprod Biol Endocrinol.* 2019;17(1):4.
140. Kim YR, et al. A systematic review: Impact of endocrine disrupting chemicals exposure on fecundity as measured by time to pregnancy. *Environ Res.* 2019;171:119–33.
141. Pivonello C, et al. Bisphenol a: An emerging threat to female fertility. *Reprod Biol Endocrinol.* 2020;18(1):22.
142. Practice Committee of American Society for Reproductive Medicine. Vaccination guidelines for female infertility patients: A committee opinion. *Fertil Steril.* 2013;99(2):337–9.
143. Chen F, et al. Effects of COVID-19 and mRNA vaccines on human fertility. *Hum Reprod.* 2021;37(1):5–13.
144. Ichiyama T, et al. Analysis of vaginal and endometrial microbiota communities in infertile women with a history of repeated implantation failure. *Reprod Med Biol.* 2021;20(3):334–44.

145. Kyono K, et al. A pilot study and case reports on endometrial microbiota and pregnancy outcome: An analysis using 16S rRNA gene sequencing among IVF patients, and trial therapeutic intervention for dysbiotic endometrium. *Reprod Med Biol.* 2019;18(1):72–82.
146. Palmer KT, et al. Pregnancy: Occupational aspects of management: Concise guidance. *Clin Med.* 2013;13(1):75–9.
147. Clark AM, et al. Weight loss results in significant improvement in pregnancy and ovulation rates in anovulatory obese women. *Hum Reprod.* 1995;10(10):2705–12.
148. Clark AM, et al. Weight loss in obese infertile women results in improvement in reproductive outcome for all forms of fertility treatment. *Hum Reprod.* 1998;13(6):1502–5.
149. Homan G, Litt J, Norman RJ. The FAST study: Fertility ASsessment and advice targeting lifestyle choices and behaviours: A pilot study. *Hum Reprod.* 2012;27(8):2396–404.
150. Legro RS, et al. Randomized controlled trial of preconception interventions in infertile women with polycystic ovary syndrome. *J Clin Endocrinol Metab.* 2015;100(11):4048–58.

35

HUMAN REPRODUCTION ACROSS THE LIFE COURSE AND THE TOTAL ENVIRONMENT

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The exposome

Introduction to the exposome and routes of exposure

From the food individuals eat to the air they breathe, the *exposome* encompasses the totality of human environmental conditions, societal environments, and unique non-genetic influences that all contribute to the risk of disease (Figure 35.1). Before conception even occurs, the environment—defined broadly to include the social, built, and natural environments— influences an individual's health from gametes to conception and throughout life. Environmental exposures to harmful chemicals, including endocrine disrupting chemicals (EDCs) and related mixtures, primarily occur in the natural, built, and individual environments that are shaped by cultural and societal conditions. EDCs are non-persistent exogenous chemicals that can impair reproductive health and precipitate adverse birth outcomes by interfering with hormonal action. In recent years, EDCs have grown increasingly ubiquitous in the natural-built environment and human body with exposure further magnified by cultural and social environments.

Exposure to EDCs typically occurs through three primary routes of exposure: (i) dermal contact and absorption of personal care products (e.g. lotion) through the skin; (ii) ingestion and absorption via the digestive tract, commonly from plastic cutlery, plastic storage containers, plastic water bottles, or the consumption of processed foods; and (iii) inhalation of contaminated air and absorption along the respiratory tract of hairspray, cleaning supplies, cigarette smoke, or other pollutants that are aerosolized and eventually dispersed by atmospheric and oceanic currents [1–3]. Daily exposure to EDCs is not singularly exclusive to dermal, digestive, or respiratory toxicants as they routinely happen concurrently. Even in utero, fetal exposure to EDCs is

possible through transplacental transition. Likewise, exposure among infants and toddlers can be intensified by hand-to-mouth behaviour.

Following exposure to EDCs, the body rapidly metabolizes short-lived EDCs, however some longer-lived EDCs with low affinity for water can accumulate in adipose tissues, ultimately reaching harmful concentrations and sometimes causing irreversible damage through teratogenic and carcinogenic mechanisms. Metabolization of EDCs depends on the location and number of chlorine atoms present in each molecule, in addition to other physiologic factors that determine one's ability to metabolize substances. Generally, the smaller the number of chlorine atoms, the faster these EDCs and their mixtures are metabolized [4]. Although most EDCs are excreted through urine and faeces, the pervasiveness of EDCs in the exposome makes daily exposure nearly unavoidable and global population exposure to these harmful chemicals more frequent and concerning.

The built and natural environments

The built environment encompasses buildings, transportation, and other man-made structures that individuals frequently occupy. In the built environment, EDCs commonly hide in laminates, varnishes, paints, epoxy resins, polyvinyl chloride (PVC) water supply pipes, polyvinyl flooring, shower curtains, polyethylene terephthalate (PET), and polyvinyl acetate (PVA) [5]. EDCs are also routinely added to construction materials, prefabricated home furnishings, colourants, lubricants, adhesives, detergents, and personal care products [5]. Off-gassing by such products poses a lingering hazard through inhalation following construction; urban areas typically have higher concentrations of EDCs compared to rural areas. Other structures, wrapped in plastic (e.g. greenhouses) increase dietary exposure to EDCs and the release of these toxicants into the food chain. For example, plants grown inside plastic greenhouses have been found to have higher concentrations of brominated flame retardants (BFRs), commonly used in industrial production to reduce flammability of building materials, compared to plants grown outside [5, 6]. More so, electronic-recycling waste (E-waste) derived from the built environment has also been found to contaminate agricultural areas through groundwater and air pollution, though indoor air has been observed to be generally more polluted with these toxicants [6–10].

The built environments permeate the natural outdoor environment through the air, dust, water, and land. Millions of deaths can be attributed to outdoor air pollution, stemming from synthetic chemical gases, liquid droplets, and small particles [11]. Pesticides, herbicides, fertilizers, and other agricultural chemicals contaminate the atmosphere as gaseous volatile organic compounds (VOCs) and semi-volatile organic chemicals (SVOCs) that easily evaporate under standard temperature and pressure [11]. In addition to agricultural sprays and E-waste, the atmosphere can be contaminated with EDCs from industrial activities, consumer

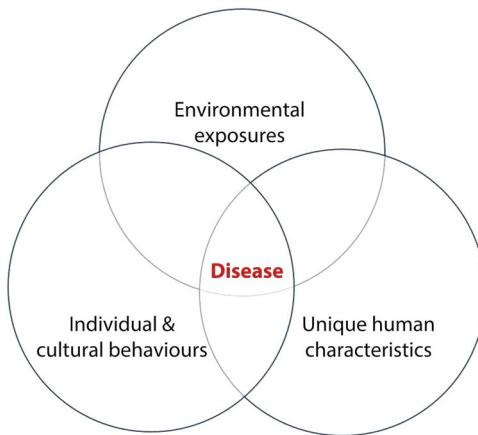


FIGURE 35.1 The exposome: Major components contributing to disease.

by-products, cigarette smoke, and diesel fumes [11]. When EDCs leach out of wastewater and sewage treatment plants, including pharmaceutical waste and livestock excretions, they contaminate drinking water resources [10]. High concentrations of EDCs have been found to seep into marine ecosystems and contribute to adverse reproductive effects and consequential population decline [12]. Given the omnipresence of EDCs, these chemicals and their mixtures interpolate the natural-built environment and pose an immense threat to many ecosystems (Figure 35.2).

Cultural, social, and individual environments

Aside from the natural-built environment, everyday exposures to harmful EDCs are intensified by daily routines, product preference, and (dietary) behaviours (Figure 35.3). Several personal care products contain EDCs, including face lotion, deodorant, face wash, shampoo, conditioner, body wash (bar and liquid soap), toothpaste, floss, cosmetics, perfume, cologne, shaving cream, contacts, contact solution, and other personal hygiene products. Some products can become aerosolized, such

as hairsprays, perfume, or household cleaning sprays, exposing the population to EDCs through inhalation and dermal absorption. Adverse health impacts to reproductivity and reproductive cancers, namely breast and testicular cancer, have been linked to consumer products contaminated with EDCs that are absorbed through the skin [13, 14]. Additionally, females have been found to have higher concentrations of urinary bisphenol A (BPA), a well-studied EDC, demonstrating the potential intensified risk of exposure from personal care products more commonly observed in women and men [15].

Dietary exposures to EDCs can occur from plastic storage containers or plastic wrap, canned food, plastic utensils, plastic linings of disposal cups and takeout containers, plastic water bottles, baby bottles, and more. EDCs are not capable of covalently bonding with plastic or canned containers, therefore direct sunlight and warm temperatures, acidic foods (the more acidic the more migration), or long storage periods promote leaching of EDCs from their containers into food and beverages [6]. Several EDCs are also lipophilic, meaning they have a great affinity for

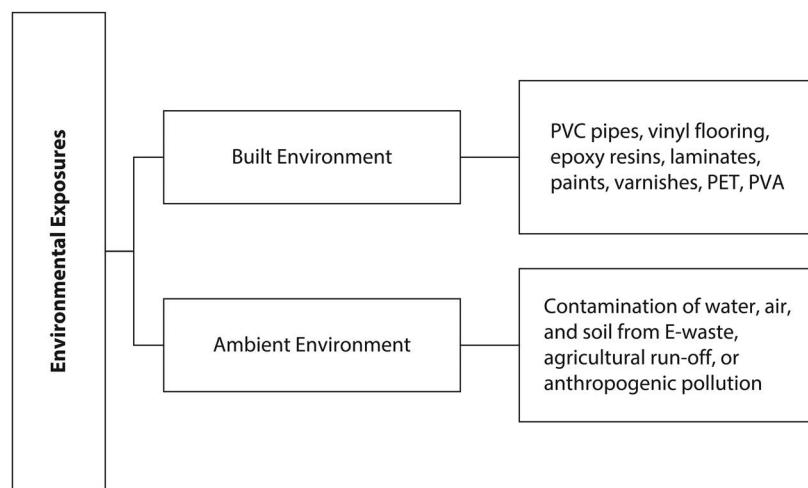


FIGURE 35.2 Main routes of exposure to EDCs in the natural-built environment.

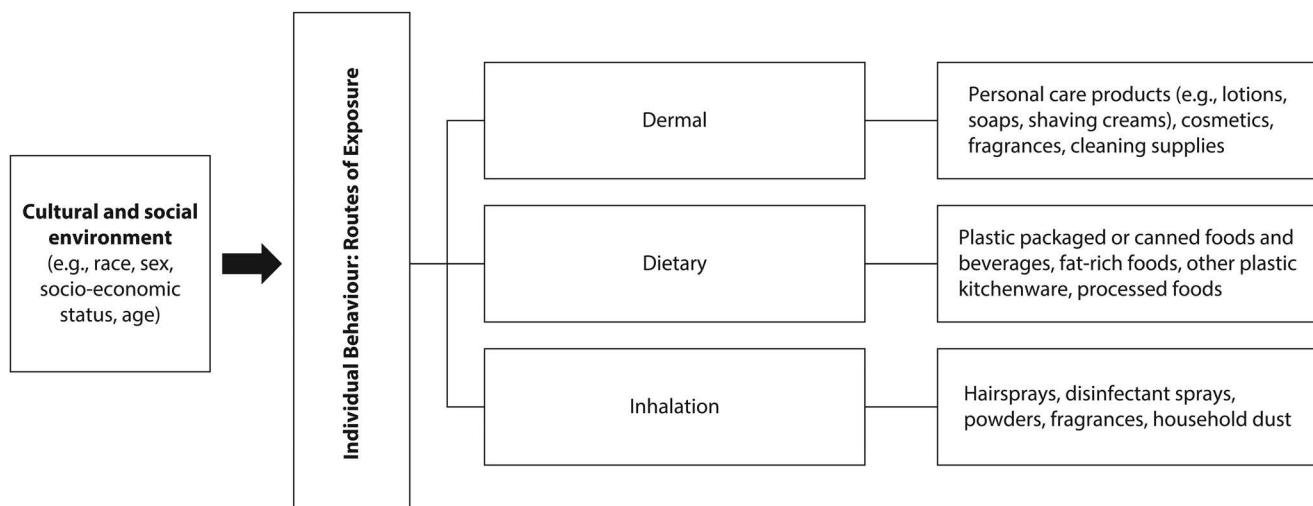


FIGURE 35.3 Possible primary routes of exposure related to cultural and social environments.

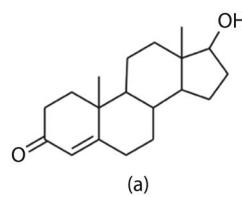
fats and oils, and can be absorbed and stored in fish, meat, dairy, breast milk, and other fat-rich foods. Fast foods also tend to have added flavours, hydrogenated oils, dyes, or hydrolyzed proteins that increase EDC content [6]. When consumed, these toxicants are absorbed by the digestive tract and eventually circulated throughout the body [3]. Dental sealants and composites might also increase the concentration of EDCs ingested by the body.

Behavioural and dietary differences between cultures also further intensify exposure to EDCs and exacerbate existing health disparities. Studies stratified by racial/ethnic line have consistently found that non-Hispanic black women (during pregnancy or the prenatal period) have higher concentrations of certain EDC metabolites linked to adverse health outcomes (e.g. preterm birth) compared to non-Hispanic whites and Mexican Americans [16–19]. Other studies have observed higher exposures to EDCs among Mexican Americans, non-Hispanic blacks, and low-income groups, specifically for EDCs that promote metabolic diseases (e.g. diabetes) [20]. These variances in EDC concentrations are hypothesized to be due to differences in preferred personal care products or cosmetics that may be influenced by hair type, skin colour, or other racial/ethnic differences [21, 22]. In addition to racial/ethnic groups, additional disparities found in the cultural and societal environment have been observed for sex, socio-economic status, and age [19].

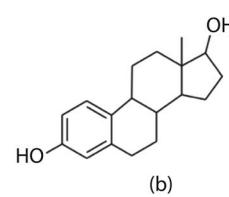
Another source of everyday EDC exposure often found within the natural-built environment includes electronic devices, such as cell phones and laptops, that emit radio frequency electromagnetic radiation (RF-EMR) and often contain EDCs to reduce flammability. While associations between RF-EMR exposure and adverse reproductive health outcomes have yielded inconsistent results and remain debatable, there have been negative associations reported for both sexes. For males, exposure to radio frequency electromagnetic waves (RF-EMW) from cell phones has been shown to decrease sperm motility and viability after one hour, and increased duration of cell phone use has been negatively associated with semen quality [23, 24]. Among females, RF-EMR exposure has been linked to decreased ovarian follicle counts in rats and decreased fetal cardiac output among pregnant women exposed to cell phones [25, 26]. Aside from RF-EMW, several electronic devices are manufactured with EDCs, such as BFRs. While the data is lacking, BFRs have been shown to have neurotoxic effects and impair reproductive health [27]. Some studies have found associations between BFRs and adverse birth effects such as low birthweight [27]. As technology use becomes more widespread, additional studies investigating the effects of electronic devices on reproductive health are needed.

Chemicals

While understanding the many factors that influence exposure patterns is fundamental, the type of EDC that someone is exposed to also matters when anticipating potential health effects. More than 80,000 potentially toxic chemicals in the environment today remain unregulated by the Environmental Protection Agency (EPA) [2]. Endocrine-disrupting capabilities have been identified in many naturally occurring and synthetic chemicals, but their true effects are still largely unknown. Some EDCs are persistent and remain in the environment (food chain), whereas others are non-persistent and are rapidly metabolized. Many of these EDC toxicants have chemical structures that resemble oestrogens, androgens, or other hormones—allowing them to easily interfere with hormonal signalling (Figure 35.4).



(a)



(b)

FIGURE 35.4 Hormone structures: (a) oestrogen (17β-Oestradiol) and (b) androgen (testosterone) [3, 29].

Examples of common EDCs with endocrine-disrupting properties associated with adverse reproductive health outcomes include BPA, phthalates, polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), chlorinated dioxins, heavy metals (Pb, Hg, Cd, As, Ni), polybrominated diphenyl ethers (PBDEs), and triclosan. These chemicals impair male and female reproductive health, resulting in reduced fertility, pregnancy loss, poor semen quality, among other potential adverse health effects (Table 35.1) [1, 30–44].

Robust epidemiologic studies have also observed adverse birth outcomes, including preterm birth, infant hypospadias, and altered birthweight. While controversial, a growing body of evidence alludes to the potential of dioxin-like PCBs contributing to the disease progression of endometriosis [45, 46]. The health effects of EDCs often extend beyond the reproductive system to also act as immunotoxins, hepatotoxins, neurotoxins, and carcinogens [3].

Short-lived chemicals

Non-persistent chemicals are typically less lipophilic and do not tend to bioaccumulate in the body. Instead, these EDCs are rapidly metabolized and excreted as urine or sweat within hours (e.g. BPA is estimated to have a half-life of around six hours) [17, 48, 49]. Amidst their quick metabolism, Calafat et al. detected BPA in 92.6% of urine samples from 2517 participants ≥ six years old in the United States (US) with recurrent exposure [15]. Examples of short-lived EDCs include phenols (bisphenols and parabens) and phthalates (Table 35.2). Alternatives to short-lived EDCs, such as bisphenol S (BPS) and bisphenol F (BPF), which have recently entered the consumer market to replace BPA, are understudied and are anticipated to have comparable toxicological effects based on their similar chemical structures. Even though short-lived EDCs are rapidly metabolized, due to their omnipresence as plasticizers and preservatives, constant exposure allows these chemicals to remain in biological systems and impair reproductive health [50].

Long-lived chemicals

Persistent EDCs primarily include organochlorine pollutants (POPs), which tend to be lipophilic and hydrophobic, allowing them to bioaccumulate in the environment. These chemicals are often stored in fat-rich foods (e.g. fish, meat, dairy) and eventually enter human bodies through dietary exposure. Examples of long-lived chemicals shown to have endocrine-disrupting mechanisms of action include PCBs, OCPs, chlorinated dioxins, and per fluorinated chemicals (PFCs) (Table 35.3) [52–59]. Some chemicals, like PFCs can be amphiphilic, meaning that their chemical structure has both lipophilic and hydrophilic characteristics. While typical use may differ, these lipophilic chemicals are also commonly manufactured to insulate electronics, lubricate automobiles or other devices, increase durability and inflammability,

TABLE 35.1 Summary of Potential Adverse Effects from Exposure to Common EDCs [1, 30–44]

Chemical	Potential Adverse Effects: Females	Potential Adverse Effects: Males	Potential Adverse Birth Outcomes
Bisphenols A (BPA)	Oocyte chromosomal abnormalities, pregnancy loss, breast cancer, infertility, endometriosis, polycystic ovary syndrome	Disrupts spermatogenesis, lowers semen quality and sperm concentration, prostate changes, sperm abnormalities	Birthweight, increased head circumference, delayed development and transport of the embryo
Phthalates (PAEs)	Irregular menstrual cycles, lower fertility or infertility, premature ovarian failure, anovulation, pregnancy loss	Decreased semen quality and sperm count, infertility, promotes testicular dysgenesis syndrome, influences pubertal timing	Preterm birth, preeclampsia, infant cryptorchidism, infant hypospadias, shorter anogenital distance
Polychlorinated biphenyls (PCBs)	Decreased fecundability, decreased lactation, irregular menstrual cycles, endometriosis, ovarian cysts, vaginal adenocarcinoma, infertility	Decreased semen quality and sperm concentration, sperm abnormalities, testicular and prostate tumours, infertility	Impaired fetal brain development, feminization of male offspring, cognitive and behavioural deficits
Organochlorine pesticides (OCPs)	Irregular menstrual cycles, reduced fertility, pregnancy loss, reduced fecundability, endometriosis	Decreased semen quality and sperm concentration, subfertility (ability of female partner to become pregnant)	Preterm birth, prolonged time to pregnancy, stillbirths, developmental deficits
Perfluorinated chemicals (PFCs)	Irregular menstrual cycles, reduced fertility and fecundity, breast cancer, early menarche, polycystic ovary syndrome	Possibly altered male fecundity, semen quality (disruption during maturation)	Low birthweight, gestational diabetes, neurodevelopmental disorders
Heavy metals (Pb, Hg, Cd, As, Ni)	Pregnancy loss, reduced fertility, irregular menstrual cycles, increased preterm labour, endometrial cancer, endometriosis, breast cancer	Abnormal sperm, reduced fertility, decreased semen quality, changes in reproductive steroidogenesis	Impaired fetal brain development, preterm birth, stillbirths, hypotrophy
Polybrominated diphenyl ethers (PBDEs)	Reduced fecundability, delayed menarche, hypothyroidism	Decreased semen quality, hormonal changes (testosterone), non-descending testes and penile malformations	Developmental neurotoxicants

and exterminate unwanted agricultural pests. Due to their longevity, these chemicals can travel great distances to expose different populations, some of which have banned such pollutants, to become global toxicants.

Summary

While many short-lived and long-lived EDCs remain unregulated, even more understudied chemicals with endocrine-disrupting properties continue to increasingly enter the environment and, in turn, the exposome. Beyond the natural-built environment and the cultural and social environment, the timing of exposure to these short-lived and long-lived chemicals should be considered. Identifying time periods and/or time windows where individuals

are the most susceptible to harmful exposures offers a life course perspective and provides clinicians, researchers, and individuals with the opportunity to more effectively intervene and prevent these insults on current and future generations.

Windows of vulnerability and reproductive health

Life course epidemiology

Exposure to EDCs and other environmental pollutants is inevitable, however recognizing periods where these exposures inflict the most damage is critical for developing interventions,

TABLE 35.2 Examples of Short-Lived Chemicals with Endocrine-Disrupting Properties [3, 54, 51, 55]

Chemical	Example Structure	Chemical Properties	Typical Use
Bisphenol A (BPA)		Hydrophilic, lipophobic, oestrogenic, anti-androgenic	Plasticizer (shape and flexibility)
Short-Lived Chemicals	Phthalates		Hydrophilic, lipophobic, oestrogenic, anti-androgenic Plasticizer (durability)
	Parabens		Lipophilic, moderate hydrophobicity, androgenic/anti-androgenic Plasticizer (cosmetics, preservatives, pharmaceuticals)

TABLE 35.3 Examples of Long-Lived Chemicals with Endocrine-Disrupting Properties [3, 52–59]

Chemical	Example Structure	Chemical Properties	Typical Use
Long-Lived Chemicals	Polychlorinated biphenyls (PCBs)	Lipophilic (water solubility decreases with increased Cl), anti-oestrogenic (planar), oestrogenic (coplanar)	Insulators and electronics (capacitors and transformers), plasticizer, paint, lubricants
	Organochlorine pesticides (OCPs), DDT	Lipophilic, hydrophobic, oestrogenic, anti-oestrogenic, androgenic, and anti-androgenic, depending on the structure	Herbicides, insecticides, fungicides, rodenticides, and other agricultural uses
	Chlorinated dioxins	Lipophilic, hydrophobic, oestrogenic, anti-androgenic	Herbicides, pesticides, chlorine bleaching, smelting, and other manufacturing processes
	Perfluorinated chemicals (PFCs), PFOA	Amphiphilic (lipophilic and hydrophilic), oestrogenic, anti-androgenic	Added to a variety of products to reduce stains, grease, and water damage; firefighting materials and other industries

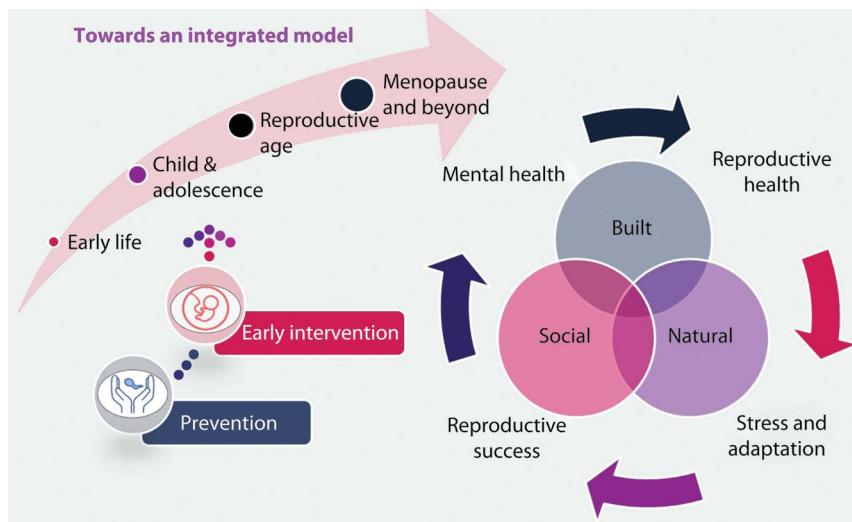
fine-tuning preventive medicine strategies, and improving reproductive health outcomes. During sensitive time periods of development certain environmental exposures can pose severe and often irreversible effects on systems, organs, tissues, and cells—ultimately impairing individual reproductive health, assisted reproductive technology (ART) outcomes (most commonly *in vitro* fertilization), and offspring health. It is important to consider environmental exposures over an individual's life course to evaluate the potential reproductive health consequences of such encounters [60]. In addition to awareness of the timing of exposures, preventive strategies and early interventions need to be implemented during vulnerable windows that consider built, social, and natural factors to promote healthy reproduction, successful ART outcomes, and offspring well-being (Figure 35.5).

Using a more integrated model, reproductive success is also contingent on an individual's mental health, reproductive health,

and ability to adapt to stressors within the natural-built and social environment. Through the application of a more comprehensive approach, life course epidemiology can be utilized to improve the reproductive success and, in turn, reproductive health of an individual and their offspring.

Preconception window: Life course exposure and paternal contribution

The preconception window represents the time period before conception. For males, the relevant sensitive preconception window usually consists of three months prior to the fertilization, as this is the time window for spermatogenesis, which lasts roughly 74 days [61]. While for females, gametes start to grow and develop when the female is in the womb of her mother; primordial follicles stay dormant during childhood. Following menarche, follicles begin to mature periodically in two or three waves per month and completely develop after three or four months

**FIGURE 35.5** An integrated model to address environmental exposures throughout an individual's life.

[62, 63]. The maturity of the dominant follicle, which is then ovulated and fertilized, usually takes about a month. The window of gametogenesis is the most direct preconception time period since it is directly related to gametes development. However, exposures before gametogenesis are also able to influence reproductive success through other mechanisms not directly related to the maturation of oocytes and spermatozoa.

The relevant preconception windows exceed the period of gametogenesis and should be viewed over the life course [64]. Importantly, in utero environmental exposures can influence embryo programming, which can impact early oogenesis and follicle formations and can also influence the development of endocrine systems, for both males and females, that are crucial for healthy reproduction [65, 66]. For example, daughters born to women who took diethylstilboestrol (DES) during pregnancy are at higher risk of developing clear cell adenocarcinoma at young ages [67, 68]. Moreover, exposure from past generations can influence the reproductive health of the offspring. For example, a grandmother's environmental exposures during pregnancy could influence a grandchild's reproductive health before the oocyte (that contributes to the future grandchild) begins developing in the womb of the grandmother [69].

Research has shown that both maternal and paternal preconception exposures to EDCs are associated with adverse birth outcomes [70]. Although females carry the fetus throughout development, paternal exposure to environmental pollutants can alter the sperm epigenome—resulting in deleterious reproductive health outcomes for the offspring [71]. Importantly, paternal preconception exposures play an important role in birth outcomes and offspring health, though this is often overlooked in the science of perinatal health. Although the mechanisms are still unclear and warrant more research, hypotheses include epigenetic changes in imprinted genes of gametes, which can bypass the epigenetic reprogramming in early embryo development, and then exert influences on pregnancy outcomes and offspring well-being [72].

Prenatal window

It is well-established that in utero exposures to environmental pollutants are associated with pregnancy loss and adverse birth outcomes in both ART pregnancies and in naturally conceived pregnancies. For example, prenatal exposure to phthalates has been consistently associated with an increased risk of preterm birth in both sub-fertile and the general fertile population [73–76]. Certain vulnerable windows exist within the prenatal period, since physiologic systems and other essential functions of the embryo/fetus develop at different pregnancy stages—the first few gestational weeks are critical for the development of essential organs. Hazardous exposures, such as teratogens, in the first few gestational weeks could lead to pregnancy loss or congenital diseases such as neural tube defects. Vulnerable windows differ for different types of environmental exposures depending on the mechanisms of how such exposures influence the development of the embryo or fetus. For example, increasing evidence shows that di(2-ethylhexyl) phthalate (DEHP) exposure during the third trimester has a higher impact on increased preterm birth risk compared with the other two trimesters of pregnancy [75]. It is hypothesized that DEHP can lead to increased oxidative stress, which is postulated as one of the triggers for preterm birth [77]. Investigating the vulnerable prenatal windows for different environmental exposures is currently an active area of research.

Summary

Given that (both maternal and paternal) exposures experienced during multiple windows of susceptibility may influence overall reproductive health of an individual and their offspring, a life course approach is well suited for understanding potential threats to reproductive health. Researchers are actively investigating the consequences of exposure to toxicants during susceptible windows and moving towards a more integrated approach to safeguard reproductive health. Understanding the potential mechanisms that EDCs and other environmental pollutants use to promote adverse reproductive health effects across an individual's lifespan will complement current evidence of vulnerable windows and increase the preservation of the reproductive health of current and future generations.

Mechanisms of action

Endocrine disruptors in biological systems and transgenerational inheritance

Wildlife biologists were among the first to make observations that chemicals and their mixtures were capable of interfering with hormone signalling to the point of altering behaviour, which provided foundational evidence to support endocrine-disrupting mechanisms triggered by dichloro-diphenyl-trichloroethane (DDT), the first recognized endocrine disruptor. In Rachel Carson's *Silent Spring* (1962) implications of widespread applications of DDT and resulting ecological disturbances and health consequences were unveiled to the public eye [78]. Since Carson's initiative, a growing body of literature has examined the mechanisms by which EDCs impact several organ systems, namely the reproductive system [79–81]. While the eventual ban of DDT decreased the presence of this endocrine disruptor in the biosphere, other endocrine disruptors remain pervasive in the environment and in the human body.

In the human body, EDCs largely disrupt molecular interactions and cellular signalling necessary to restore and maintain homeostasis (equilibrium). EDCs are exogenous chemical compounds or mixtures capable of interfering, mimicking, blocking, or otherwise altering aspects of normal hormonal action through a variety of mechanisms, including but not limited to the interference of protein synthesis, secretion, cellular transport, and receptor binding [81–85]. EDCs can act as an agonist (initiator) or antagonist (inhibitor) to oestrogen, androgen, and other nuclear or membrane-bound hormone receptors to enhance or block cellular and systemic hormonal processes (Figure 35.6) [86]. These molecular modifications, complemented by epigenetic alterations, influence endocrine communication by shifting hormone levels in the blood—causing dysfunction of reproductive, immune, neurological, and metabolic systems. Environmental exposure to EDCs during these developmental periods can lead to reprogramming of the epigenome through receptor-mediated or non-receptor-mediated pathways and altered gene expression that persist for generations [87].

Epigenetic modifications and oncogenesis

Though there is strong evidence to support the association between EDCs and adverse birth outcomes, little is known about how these chemicals directly influence genes to produce such outcomes. While human studies are lacking, prenatal exposure to phthalates and postnatal outcomes have been associated with epigenetic modifications such as placental DNA methylation and altered birthweight [87]. Another study that recruited

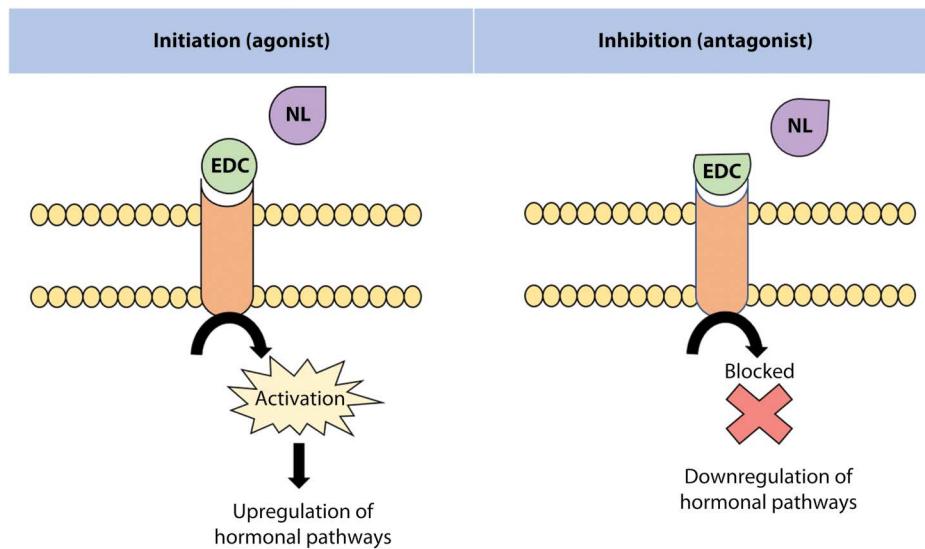


FIGURE 35.6 Examples of activation and inhibition of natural ligand (NL) by EDCs.

infant–mother pairs to investigate phthalate exposure in utero, found increased methylation of CpG sites (cytosine and guanine sites that are common targets for methylation) in cord blood [88]. DNA methylation has also been observed in several EDC-associated CpGs that may contribute to metabolic disorders (i.e. obesity, type II diabetes, and insulin resistance) [89]. Other common epigenetic modifications include mRNA polyadenylation and histone modification, which damage DNA, detrimentally affect post-transcriptional regulation, and contribute to adverse reproductive outcomes by disturbing oocyte maturation, among other important processes [86].

Epigenetic modifications that damage DNA also promote malignant differentiation of cells that can lead to reproductive cancers. For example, BPA, DDT, and PCBs exposure has been linked with incident breast cancer cases globally [90]. BPA, DDT, and PCBs alter cellular pathways involved in cell proliferation and cell death. These chemicals act through genotoxic and carcinogenic mechanisms that cause epigenome changes, suppression of the immune system, and promotion of oxidative stress (a form of DNA damage that increases free radicals) [90]. DDT and PCBs have been shown to contribute to chronic inflammation, also increasing the risk of developing cancer [90]. Characteristic of other environmental exposures, some EDCs have a latency effect where the consequences following exposure can manifest later in life (e.g. exposure in utero may increase the risk of hormone-sensitive cancers).

Dysfunction of the immune system and metabolic diseases

Once thought to be relatively independent systems, immune–endocrine interactions are essential to adequately fight infections and prevent metabolic illnesses [91]. Following initiation of the innate immune response and antigen (pathogen) recognition, the immune system initiates rapid proliferation of several immune cells that can phagocytose or kill invading pathogens. During this response, T cells, macrophages, among other immune cells, become dependent on glycolytic products instead of oxidative phosphorylation, resulting in an increased demand

for energy (glucose) [92]. The endocrine system is responsible for shifting nutrient pathways to accommodate the needs of immune cells and supply sufficient glucose for these cells to properly fight off infections and inflammation. However, when disruptions in endocrine pathways necessary for glycaemic control persist, the body becomes vulnerable to metabolic diseases, like type II diabetes, among other immunocompromised conditions.

Few risk assessments have explored the effects of EDCs on the immune system; however, some wildlife studies have shed light on EDCs impact on the innate immune system, related gene regulation and transcription, and oxidative stress that contribute to weakened immune responses. Early toxicity studies explored the effects of DDT in animal models and observed suppression of the primary humoral immune response [92]. Other early investigations of EDCs found potential immunomodulation in animal models that resulted in immunosuppression and thymic atrophy [14, 93–96]. More recent animal studies in rats treated with methoxychlor (MXC), a pesticide, during the perinatal and pre-pubertal stages, witnessed decreased antibody-mediated immune response, lower epididymal sperm counts, and reduced testis weight [95]. Other EDCs that increase inflammation can directly influence the reproductive system and promote the development of metabolic diseases [96].

Oestrogen receptors are commonly expressed by many immune cells involved in the inflammatory response. For example, BPA acts as both an oestrogen receptor agonist and antagonist, increases inflammation, and contributes to chronic inflammation. BPA enhances the proliferation of immune cells such as B lymphocytes and thymocytes, in addition to decreasing the number of regulatory T cells (which help reduce inflammation so that the body can return to homeostasis) [96]. Besides BPA, many EDCs remain understudied in spite of evidence that endocrine disruption can cause deregulation of the immune system and contribute to metabolic diseases. Aside from metabolic and immune toxicity, some EDCs have obesogenic, carcinogenic, hepatotoxic, nephrotoxic, and other systemic effects. Heavy metals and other environmental pollutants that can impact the endocrine system

also disrupt the nervous system and impair learning and memory pathways, including basic motor function [3].

Effects of EDCs on the reproductive system

Besides immunotoxicity and other systemic effects, epigenetic modifications lead to the disruption of hormonal pathways that have a broad range of adverse consequences on reproductive health and birth outcomes. The reproductive system is highly dependent on the endocrine system; several hormonal pathways are essential for fertility, fecundity, proper pubertal timing, menstrual cycles, and fetal development, among other outcomes. Interference of these pathways can lead to hormone-driven reproductive diseases (e.g. endometriosis and reproductive cancers). It has been hypothesized that the feedback loops of the hypothalamus-pituitary-gonadal axis are heavily influenced by EDCs, though these mechanisms are still being unravelled [86]. Early windows of exposure and the reproductive cycle (gametogenesis and embryogenesis) are especially susceptible to toxicants since they rely heavily on steroid hormones and receptors that can be inhibited by EDCs and their mixtures.

Hormonal imbalances of oestrogen, progesterone, thyroid hormones, and other hormones can lead to improper fetal development, menstrual cycle irregularities, reduced fertility, endometriosis, pregnancy loss, decreased semen quality, reduced sperm concentration, among other hormone-driven reproductive health disorders (Table 35.4) [14, 34]. In utero and perinatal exposure to EDCs can change the concentration of luteinizing hormone (LH) via the hypothalamus, which has downstream consequences such as pregnancy loss. EDCs are thought to act via oestrogen receptor-mediated pathways that alter gene expression and normal differentiation during development of the female reproductive tract [97]. For males, EDCs disrupt testes differentiation and spermatogenesis, which encourage congenital malformations, development of hypospadias, adult reproductive disorders, among other adverse reproductive health outcomes [14]. Lastly, fetal neurodevelopment and cognitive function are impaired when EDCs interfere and interact with several thyroid receptors and alter thyroid hormone concentrations in utero. A large breadth evidence

alludes to EDCs having a substantial impact on fetal and infant health and development, especially when exposure happens during windows of vulnerability [30, 96, 97].

Summary

EDCs and their mixtures are capable of interfering with normal hormonal processes and affecting multiple physiologic systems, including the reproductive system. Due to their oncogenic, obesogenic, mutagenic, and carcinogenic effects, these chemicals pose a great threat to human health and reproductive health outcomes. Evidence of multigenerational inheritance of epigenetic modifications calls for urgent action to clarify potential reproductive health toxicants and reduce population exposure.

Environmental exposures and reproductive health

Reproductive health outcomes and associated toxicants

Several reproductive health outcomes in both males and females, including fertility and fecundability, semen quality, ART outcomes, and birth outcomes, are compromised by environmental toxicants. The deleterious outcomes following environmental exposure to common pollutants, such as air pollution, EDCs (both non-persistent and persistent chemicals), and heavy metals are summarized in Table 35.5.

Fertility, fecundability, and environmental exposures

Several environmental factors are shown to be related to reduced antral follicle counts (AFC), a marker of ovarian reserve, fecundability, and infertility in sub-fertile and fertile populations. For example, in a cohort of sub-fertile women seeking fertility treatment in Massachusetts (the Environment and Reproductive Health Study, EARTH cohort), higher residential exposure to air pollution, specifically fine particulate matter (PM 2.5), was inversely associated with antral follicle count [103]. In the same cohort, temperature was negatively associated with AFC with a 1.6% lower AFC associated with a 1°C increase in average

TABLE 35.4 Summary of Potential Mechanisms of Action for Common EDCs [1, 31, 35, 36, 47, 90, 100–102]

Chemical(s)	Examples of Potential Mechanisms of Action
Bisphenol A (BPA)	<ul style="list-style-type: none"> • Inhibition of transcription for many genes, changes in gene expression and mRNA levels • Interference with thyroid, oestrogen, and androgen receptors (mimic, antagonist, and agonist) • Selective oestrogen receptor modulator (SERM) interferes with peroxisome proliferator-activated receptors and nuclear oestrogen receptors (α and β) resulting in weak oestrogenic activity
Phthalates (PAE)	<ul style="list-style-type: none"> • Increases expression of genes related to metabolism, synthesis, hormone transport • Downregulation of thyroid receptors (TSH), upregulation of thyroid hormones (TRH) • Modulate activity of nuclear and membrane receptors (oestrogen, androgen, and peroxisome proliferator-activated receptors) as agonists or antagonists
Polychlorinated biphenyls (PCBs)	<ul style="list-style-type: none"> • Interference in oestradiol production and synthesis of transport proteins specific to hormones • Bind to oestrogen receptors and have anti-oestrogenic activity • Potentially decreases thyroid availability in the fetal brain, necessary for normal development
Polybrominated diphenyl ethers (PBDEs)	<ul style="list-style-type: none"> • Binds thyroid receptors, inhibits triiodothyrosine and interferes with metabolism of thyroid hormones • Oxidative DNA damage, mitochondrial dysfunction, apoptosis • Interferes with calcium signalling and other neurotransmitter pathways that can impair motor activity and cognition if exposure happens during pre- and or postnatal stages
Organochlorine pesticides (OCPs)	<ul style="list-style-type: none"> • Interferes with central nervous system by blocking γ-aminobutyric acid (GABA) receptors • Lipophilic properties allow it to bioaccumulate and influence several cellular processes • Some OCPs have placental toxicity, inhibit oestradiol, or lead to reduced oestrogen and progesterone

TABLE 35.5 Summary of Environmental Exposures and Related Reproductive Outcomes [115, 157, 169]

	Male and Female Fertility and Fecundability	Semen Quality	ART Outcomes	Birth Outcomes
Air Pollution				
Particulate Matter 2.5 (PM 2.5)	↓ Fecundability ↓ Antral Follicle Counts ↑ Infertility	↓	Limited Data	↑ Pregnancy Loss ↑ Preterm Birth ↓ Birthweight
Sulphate Dioxide (SO ₂)	↓ Antral Follicle Counts	↓	Limited Data	↑ Pregnancy Loss
Non-Persistent Chemicals				
Phthalates	↓ Fecundability ↓ Antral Follicle Counts ↑ Infertility	↓	↓ Number of Oocytes Retrieved ↓ Number of Mature Oocytes ↓ Number of Fertilized Oocytes	↑ Pregnancy Loss ↑ Preterm Birth ↓ Birthweight
Bisphenol A (BPA)	↑ Infertility	↓	↓ Peak Oestradiol Response ↓ Number of Oocytes Retrieved ↓ Number of Mature Oocytes ↓ Number of Fertilized Oocytes ↓ Implantation Rate	↑ Preterm Birth ↓ Birthweight
Paraben	↓ Fecundability	↓	↓ Peak Oestradiol Response ↓ Number of Mature Oocytes ↓ Live Birth	↑ Preterm Birth
Persistent Chemicals				
Polychlorinated biphenyl (PCBs)	↓ Fecundability ↑ Infertility	↓	↓ Implantation Rate ↓ Live Birth	↓ Birthweight
Per- and polyfluoroalkyl Substances (PFAS)	↓ Fecundability ↑ Infertility	↓	↓ Number of Oocytes Retrieved Limited Data	↑ Preterm Birth ↓ Birthweight
Heavy Metals (Pb, Cd, As)	↓ Fecundability	↓	↓ Number of Mature Oocytes ↓ Number of Fertilized Oocytes ↓ Implantation Rate	↑ Preterm Birth ↓ Birthweight

maximum temperature during the 90 days before ovarian reserve testing [104]. Additionally, in a Chinese study observing the general population, ambient sulphur dioxide (SO₂) exposure during oogenesis was significantly associated with lower AFC [105]. In other studies, living near a major roadway was associated with an increased risk of self-reported infertility (inability to achieve pregnancy after one year of unprotected intercourse) [106]. Similarly, average PM 2.5 exposure levels over the 60 days preceding the end of the first month of unprotected intercourse has been associated with decreased odds of achieving pregnancy during the first month [107]. Further studies have observed that one-year averaged ambient PM 2.5 exposure may be associated with decreased fecundability (longer time to pregnancy [TTP]) and increased risk of self-reported infertility among a large Chinese cohort [108].

Exposure to short-lived or non-persistent chemicals, including phthalates and phenols, has been linked to lower AFC, premature ovarian failure, fecundability, and infertility. Exposure to EDCs, such as Di(2-ethylhexyl) phthalate (DEHP), in the months preceding ultrasound assessment was associated with decreased AFC in the previously mentioned EARTH cohort [109]. A case-control study in China found that mono-isobutyl phthalate (MiBP) exposure was significantly associated with increased odds of premature ovarian failure [110]. Additionally, a cohort study of couples planning for pregnancies in Greenland, Poland, and Ukraine found female DEHP exposure was related to longer TTP [111]. Similarly, another study in US couples found male instead of female exposure to monomethyl (MMP), mono-n-butyl (MBP), and monobenzyl (MBzP) phthalates to be associated with reduced fecundability (longer TTP) [112]. Generally, women working in

occupations with potential high phthalate exposure have a greater risk of infertility. For example, a Danish study found an increased incidence of infertility treatment among women working in the plastic industry, and a separate study found an increased risk of TTP > 6 months in women with a job matrix containing probable phthalate exposure [113, 114]. Exposure to phenols, such as BPA, are also associated with infertility or impaired fecundity in females [113]. Other phenols, such as triclosan, have been associated with decreased fecundity [116]. There is some evidence suggesting that female exposure to methyl paraben (MePB) and ethyl paraben (EPB) is associated with diminished fecundability [117].

Persistent chemical exposures, including PCBs and per- and polyfluoroalkyl substances (PFAS), are reported to also be related to infertility and reduced fecundability. Several studies reported an association between PCB exposure and longer TTP. For example, women exposed to high concentrations of PCBs in the 1978–1979 Taiwanese incident of cooking oil contamination were found to have reduced fecundability compared to unexposed women [118]. Additionally, two prospective studies in the general US population found total PCBs concentrations in female serum are related to reduced fecundability [119, 120]. Several European cohorts have also observed positive associations between maternal exposure to perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), and perfluorononanoic acid (PFNA) and TTP [121–123]. Importantly, a preconception cohort of couples planning a pregnancy found an association between PCBs exposure in both males and females and increased TTP [124]. In this same study, female exposure to perfluorooctane sulfonamide (PFOSA) was associated with reduced fecundability, though the detection rate of this compound was very low (10%) [124].

While several non-essential metals, like cadmium, arsenic, and mercury, are known to be harmful to physiologic systems, there is currently limited data and often conflicting results when investigating heavy metal exposure and fertility. Among those limited studies, in a large US cohort study (LIFE study), both female blood Cd and male blood Pb were associated with reduced fecundability among couples [125]. However, another study conducted among 99 women residing in New York state revealed no obvious influences of As, Cd, or Pb on TTP [126].

Environmental exposures and semen quality

Human sperm count has been reported to be decreasing over the past several decades, but whether these decreases impact male fertility is debatable; however, environmental exposures are thought to play a role [127, 128]. Semen quality, including sperm count, concentration, morphology, and motility, is directly related to male fertility. Sperm quality can also impact offspring health through epigenetic changes as shown through multigenerational research on paternal exposures. Common air pollutants (PM 2.5 and PM 10, nitrogen oxides [NO_x], ozone [O_3], and sulphur dioxide [SO_2]) have been related to changes in several parameters of semen quality [129]. Consistent associations have been observed across epidemiological studies for increased sperm DNA fragmentation, abnormal morphology, and decreased motility. More so, exposure to heat stress and increased scrotal heat through occupations (e.g. bakers, steel workers) or lifestyle (e.g. hot bath, cycling) are also reported to be associated with decreased sperm count and quality [130–132]. In the EARTH cohort, primarily wearing boxer style underwear was related to higher sperm concentration and count compared to men who wore tighter underwear [133].

Cross-sectional and cohort studies have consistently shown associations between non-persistent chemicals (phthalates and BPA) and reduced semen quality, including decreased sperm concentration and motility, and increased abnormal sperm morphology, among fertile males and males seeking fertility treatments [134]. There is some evidence that paraben exposure decreases sperm motility and concentration, while increasing abnormal sperm morphology [134]. More so, urinary paraben concentrations have been associated with sperm DNA damage in male partners from the EARTH study [135]. Among persistent chemicals, PBDEs exposure measured in serum, hair, or seminal fluid has been associated with decreased sperm concentration and motility [134]. Existing evidence supports associations between serum DDT/DDE levels and poor semen quality, particularly reduced sperm motility [134]. Not surprisingly, exposure to pesticides in agricultural work, intake of pesticide residuals, and urinary pesticide biomarkers have been associated with decreased semen quality [136, 137]. Some evidence links PCB exposure to reduced semen quality [134]. Currently, there is limited data on PFAS and semen quality [138, 139]. However, one study from Denmark showed lower percentage of morphologically normal sperm in men who had high combined PFOA and PFOS levels as compared with those who had low levels [140].

Occupational and environmental exposure to heavy metals, including Hg, Cd, Pb, and As, have been associated with decreased semen quality and altered reproductive hormone levels [141]; however, like fertility, studies investigating exposure to heavy metal and semen quality remain limited.

Environmental exposures and ART outcomes

Given that there is limited data on air pollution and ART outcomes, most of the findings are generated by the US EARTH study.

Among the few existing studies, prenatal exposure to PM10 and nitrogen dioxide (NO_2), especially exposure in the early weeks of pregnancy, were related to increased risks of early pregnancy loss and decreased probability of live birth for pregnancies conceived with ART [142, 143].

Current evidence linking phthalates exposure and ART outcomes is inconclusive, possibly due to heterogeneous study designs and differences in population characteristics (age, infertility, and treatment) [143–146]. Periconception DEHP exposure has been negatively related to number of oocytes retrieved, number of mature oocytes, and number of fertilized oocytes, while there are inconsistent results for DEHP exposure and number of top-quality embryos. Limited data is available for phthalates substitutes, such as di(isonyl) cyclohexane-1,2-dicarboxylate (DINCH), however findings from the EARTH cohort showed negative associations between concentrations of a DINCH metabolite (MHiNCH) and peak oestradiol and number of total oocyte yields [147]. Conversely, another study in Israel found no relationships between DINCH metabolites and IVF outcomes [146]. Among phenols, BPA exposure has been associated with a reduced peak oestradiol response during IVF procedure [148]. Higher maternal urinary BPA concentrations are associated with decreased ovarian response, decreased peak serum oestradiol, fewer oocytes retrieved, fewer normally fertilized oocytes, and reduced implantation rates [149–151]. Urinary triclosan were negatively associated with top-quality embryos and implantation rate, and number of oocytes retrieved among women undergoing IVF [152, 153]. Notably, paternal paraben exposure was associated with decreased probability of live birth for intrauterine insemination (IUI) protocols [154].

Serum concentrations of total PCBs is associated with reduced probabilities of implantation and live birth among women undergoing IVF/ICSI treatments [155]. A small study also found follicular fluid BDE 153 (a type of PBDE) to be associated with reduced embryo implantation [156]. Similarly, PBDE is related to decreased probability of clinical pregnancy only in non-White women in the EARTH cohort [155]. In the EARTH cohort, female urinary concentrations of the sum of the organophosphate flame retardant (OPFR) metabolites were associated with reduced probability of successful fertilization, implantation, clinical pregnancy, and live birth, and increased risk of pregnancy loss [158, 159]. Additionally, paternal urinary concentrations of BDCIPP (a type of OPFR) were associated with reduced fertilization in this same cohort [160]. Currently, there is only a handful of studies on PFAS and ART outcomes with heterogeneous study design and small sample sizes [161–165]. Of those studies, follicular PFHxS concentration negatively related to follicle count. Additionally, perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) were related to decreased blastocyst formation rate among women undergoing ART [164].

Relationships between heavy metal exposure and ART outcomes are inconsistent, however some studies suggest relationships between follicular fluid Cr concentrations and decreased number of mature oocytes, maternal urinary Cd concentration and a lower probability of oocyte fertilization, and maternal serum Cd level and reduced implantation probability [166–168].

Pregnancy, birth outcomes, and environmental exposures

Chronic and prenatal exposures to air pollutants have been consistently associated with increased risks of pregnancy loss, preterm birth, and low birthweight. For example, a Chinese cohort

study found prenatal exposure to sulphur dioxide (SO_2) and total suspended particles to be associated with early fetal loss [167]. A study in Iran found prenatal exposure to nitrogen dioxide (NO_2) and ozone (O_3) were associated with increased risk of pregnancy loss before 14 weeks gestation [170]. Additionally, an Italian study with lower background air pollution found particulate matter and ozone were associated with increased risks of pregnancy loss [171]. Chronic exposures to ozone (O_3) and PM2.5 air pollutants throughout pregnancy have been associated with pregnancy loss among US couples [172]. Regarding birth outcomes, a systematic review showed that ambient and household PM 2.5 were associated with reduced birthweight and increased risk of preterm birth, particularly in low- and middle-income countries [173].

Aside from air pollution, prenatal phthalates have been associated with increased risk of pregnancy loss in naturally conceived pregnancies [174–178]. Additionally, prenatal urinary DEHP concentrations are also related to increased risk of preterm birth, some studies noting the third trimester as the vulnerable window [73–76]. Limited evidence suggests that phthalates replacements, such as DINCH metabolites, are related with elevated risk of preterm birth [73]. Maternal and paternal preconception concentrations of DEHP metabolites have been associated with increased risk of preterm birth [179]. Similarly, paternal preconception urinary concentrations of DEHP metabolites (MBP, MBzP, and MiBP) were associated with decreases in birthweight among IVF-conceived singletons, whereas maternal preconception urinary BPA and monoethyl phthalate (MEP) concentrations were associated with decreased birthweight among all singletons from the EARTH cohort [71, 180]. Prenatal urinary BPA concentrations was positively associated with the risk of preterm birth with mid-to-late pregnancy as potential vulnerable windows [181–184]. There is also evidence suggesting that paraben concentrations are positively associated with risk of preterm birth though evidence is inconsistent across studies [181, 185–187]. Further evidence supports an association between maternal and paternal preconception exposure to preterm birth and birthweight [71, 188, 189]. Maternal preconception exposure to BPA was associated with preterm birth and decreased birthweight, while paternal paraben exposure is related to increased risk of preterm birth [190, 191].

Exposure to persistent chemicals, such as PCBs, is associated with decreased birthweight, but findings with preterm birth are inconsistent [190–192]. Evidence on PFAS exposure is limited; however, the Danish birth cohort, Swedish SELMA pregnancy cohort, and the C8 project have shown that prenatal PFAS exposure is linked to miscarriage [193–195]. Reduced birthweight is the most consistently reported adverse birth outcome associated with prenatal PFAS exposure [196–199]. Prenatal PFOA, PFOS, PFNA, and PFHxS exposures were consistently observed to be associated with decreased birthweight in cohorts from Denmark, Sweden, Spain, Britain, the United States, and China [200–209]. Epidemiologic evidence on prenatal PFAS exposure and gestational age or preterm birth is inconsistent, with some studies observing associations [196, 202, 206] and others observing null [205, 207, 211].

Arsenic, cadmium, and lead exposure during pregnancy have been associated with decreased birthweight and increased preterm birth [212–215]. Among the identified heavy metals, cadmium has been found to have the most distinct effects on several birth outcomes, including birthweight, small for gestational age, and crown-heel length [215].

Summary

Frequent and prevalent population exposure to air pollution (PM 2.5), various persistent and non-persistent chemicals, and some heavy metals have been demonstrated to be harmful to both male and female reproductive systems. To address these exposures, comprehensive and effective interventions that aim to reduce internal concentrations of EDCs and limit exposure to environmental toxicants need to be developed and fine-tuned to improve public health, especially reproductive health, around the globe.

Clinical interventions for environmental exposures

Introduction to clinical interventions and key windows of exposure

Despite growing interest in EDCs and robust epidemiologic evidence supporting various adverse reproductive health effects, clinical and community-level evidence-based interventions remain underdeveloped and concerningly limited during critical windows of exposure among the most susceptible populations. Well-designed interventions could potentially reduce environmental exposures to EDCs, like phthalates and phenols, when routes of exposure to exogenous and non-persistent chemicals are most probable. As previously discussed, EDCs can be found in a variety of everyday products, such as personal care products, canned or plastic-packaged food, and cleaning supplies. Yet, interventions targeting decreased environmental exposure to such chemicals remain underutilized, and the risk of adverse health outcomes associated with EDC exposure persist.

Although interventions can be applied during different stages of the human life cycle, the preconception period, when several reproductive and developmental outcomes are programmed, has proven particularly vulnerable to phthalates and phenols [36, 216–219]. Exposure to EDCs during the preconception period has been consistently associated with reduced fecundability, miscarriage, poor-quality embryos, sperm methylome alterations as a result of epigenetic mechanisms in oocytes, and reduced sperm count [218–223]. Additional studies have observed enduring epigenetic modifications following exposure to EDCs during the preconception period that result in multigenerational epigenetic inheritance [69, 98, 223–226]. While environmental exposure to EDCs during the preconception period poses several potential consequences, identifying susceptible stages provides investigators and clinicians with the opportunity to intervene during key windows of exposure to prevent adverse health outcomes and protect generational health.

The importance of male participation for intervention development

For females, exposure to EDCs may decrease fertility; lead to poor-quality embryos, damaged oocytes, and pregnancy loss; or impair fetal and infant health postpartum (birth size) [33, 226, 227]. On the other hand, exposure among males decreases sperm motility, shifts pubertal timing, impairs testicular function and seminiferous tubules, reduces sperm count, among other consequences from epigenetic modifications, including cancer in reproductive organs (e.g. prostate) [72, 154, 227, 228]. Although males don't tend to use cosmetics as regularly as females, they likely use several other personal care products, including shaving cream, body wash, and face lotion, that contribute to routine, everyday exposure to EDCs. A growing body of literature

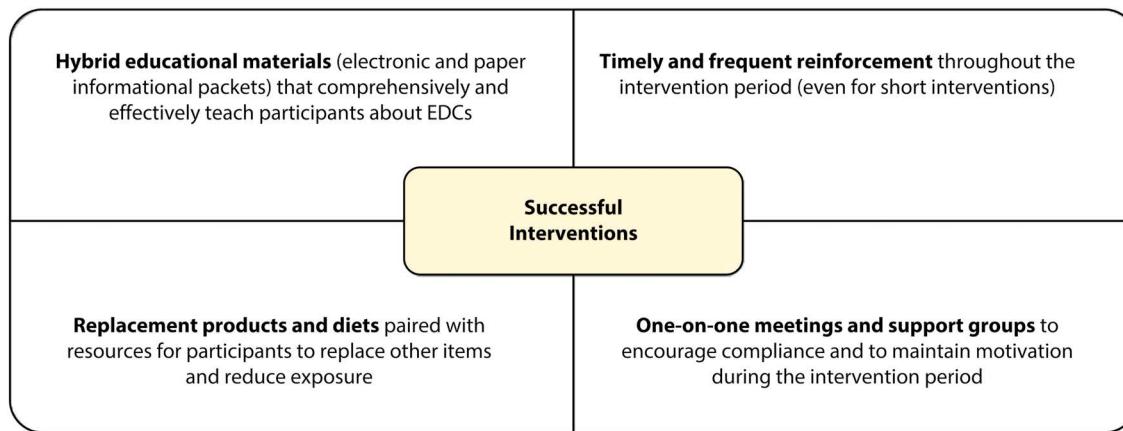


FIGURE 35.7 Successful strategies among current interventions to apply to future interventions.

supports that paternal (male) environmental exposure to EDCs contributes to several adverse birth outcomes, such as reduced sperm quality, through epigenetic modifications and alterations during spermatogenesis [70, 216, 218, 228–232]. These findings highlight the urgency to include males in clinical and community interventions to reduce adverse reproductive health outcomes among both sexes and mitigate adverse birth outcomes. Recognizing the consequences of exposure to EDCs among males and acknowledging the importance of their inclusion in future interventions is essential for the development of strategies that aim to reduce adverse effects on male reproductive health and couple-based pregnancy outcomes.

Brief overview of current interventions and successful strategies

Designing successful interventions, that see reduction in at least some EDC metabolites, is a daunting and challenging task due to the pervasiveness of these chemicals. At present, the lack of published studies has limited the development of intervention strategies based on known and predicted outcomes. After review of available EDC publications, efficacious interventions appear to share the qualities listed in Figure 35.7 [233–242]. Successful interventions tend to (i) have both electronic and paper educational materials (electronic or online materials have been shown to be more effective than paper), (ii) the research team uses timely reinforcement every few days to ensure participants avoid possible exposure to EDCs, (iii) the intervention involves replacement products and or diets where multiple routes of exposure are targeted, and (iv) the research team encourages participants to stay motivated by organizing meetings to facilitate questions and comments during the intervention period. Unsuccessful interventions tend to only focus on a single route of exposure, fail to replace multiple potential products, or forgo following up with their participants to ensure reinforcement and compliance. In the future, interventions need to be implemented to address known routes of exposure and identify new EDC exposure pathways to ultimately facilitate the development of clinical and public health guidelines. Since environmental exposure to EDCs remains a hazard outside of the preconception period, future interventions should focus on both short-term and long-term exposure reduction techniques to avoid adverse health outcomes.

Summary

Barring the comprehensive introduction of new personal care products free of EDCs like phthalates, phenols, and parabens, elimination of EDCs from everyday products remains exceptionally challenging. To fine-tune intervention strategies and address the tremendous lack of EDC interventions, larger clinical and community-based strategies need to be implemented. Paternal participation should be encouraged during such clinical interventions given their contribution to couple-based pregnancy outcomes and to better understand the effects of EDCs and other environmental toxicants on male reproductive health. Future interventions should focus on short-, mid-, and long-term exposures along with multiple routes of exposure to allow for comprehensive and adequate development of clinical and public health guidelines for all populations at risk.

Conclusion

There is an urgent need for policy development and implementation of interventions to mitigate exposure to environmental toxicants among the most vulnerable populations. With exposure to various environmental toxicants, including air pollution, non-persistent and persistent chemicals (including EDCs), and heavy metals, becoming more widespread and routine, it is important to recognize the immense impact these exposures have on current populations and future generations. Thus, adopting a life course perspective, further identifying key windows of exposure, elucidating potential mechanisms of action, investigating potential routes of exposures and other environmental factors, and designing effective interventions are core to protecting population health and promoting healthy reproduction.

References

- Matuszczak E, Komarowska MD, Debek W, Hermanowicz A. The impact of bisphenol A on fertility, reproductive system, and development: A review of the literature. *Int J Endocrinol*. 2019;2019:4068717.
- Philippat C, Bennett D, Calafat AM, Picciotto IH. Exposure to select phthalates and phenols through use of personal care products among Californian adults and their children. *Environ Res*. 2015;140:369–76.

3. Yilmaz B, Terekci H, Sandal S, Kelestimur F. Endocrine disrupting chemicals: Exposure, effects on human health, mechanism of action, models for testing and strategies for prevention. *Rev Endocr Metab Disord.* 2020;21(1):127–47.
4. Grimm FA, Hu D, Kania-Korwel I, Lehmler HJ, Ludewig G, Hornbuckle KC, et al. Metabolism and metabolites of polychlorinated biphenyls. *Crit Rev Toxicol.* 2015;45(3):245–72.
5. Sun J, Wu Y, Jiang P, Zheng L, Zhang A, Qi H. Concentration, uptake and human dietary intake of novel brominated flame retardants in greenhouse and conventional vegetables. *Environ Int.* 2019;123:436–43.
6. Sokal A, Jarmakiewicz-Czaja S, Tabarkiewicz J, Filip R. Dietary intake of endocrine disrupting substances presents in environment and their impact on thyroid function. *Nutrients.* 2021;13(3):867.
7. Hwang HM, Park EK, Young TM, Hammock BD. Occurrence of endocrine-disrupting chemicals in indoor dust. *Sci Total Environ.* 2008;404(1):26–35.
8. Rudel RA, Dodson RE, Perovich LJ, Morello-Frosch R, Camann DE, Zuniga MM, et al. Semivolatile endocrine-disrupting compounds in paired indoor and outdoor air in two northern California communities. *Environ Sci Technol.* 2010;44(17):6583–90.
9. Oziol L, Alliot F, Botton J, Bimbot M, Huteau V, Levi Y, et al. First characterization of the endocrine-disrupting potential of indoor gaseous and particulate contamination: Comparison with urban outdoor air (France). *Environ Sci Pollut Res Int.* 2017;24(3):3142–52.
10. Wee SY, Aris AZ. Occurrence and public-perceived risk of endocrine disrupting compounds in drinking water. *Npj Clean Water.* 2019;2(1):17755.
11. Darbre PD. Overview of air pollution and endocrine disorders. *Int J Gen Med.* 2018;11:191–207.
12. Street ME, Angelini S, Bernasconi S, Burgio E, Cassio A, Catellani C, et al. Current knowledge on endocrine disrupting chemicals (EDCs) from animal biology to humans, from pregnancy to adulthood: Highlights from a national Italian meeting. *Int J Mol Sci.* 2018;19(6):1647.
13. Jeong H, Kim J, Kim Y. Identification of linkages between EDCs in personal care products and breast cancer through data integration combined with Gene network analysis. *Int J Environ Res Public Health.* 2017;14(10):1158.
14. Giulivo M, Lopez de Alda M, Capri E, Barcelo D. Human exposure to endocrine disrupting compounds: Their role in reproductive systems, metabolic syndrome and breast cancer. A review. *Environ Res.* 2016;151:251–64.
15. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. *Environ Health Perspect.* 2008;116(1):39–44.
16. Kahn LG, Philippat C, Nakayama SF, Slama R, Trasande L. Endocrine-disrupting chemicals: Implications for human health. *Lancet Diabetes Endocrinol.* 2020;8(8):703–18.
17. Calafat AM, Ye X, Wong LY, Bishop AM, Needham LL. Urinary concentrations of four parabens in the U.S. population: NHANES 2005–2006. *Environ Health Perspect.* 2010;118(5):679–85.
18. Silva MJ, Barr DB, Reidy JA, Malek NA, Hodge CC, Caudill SP, et al. Urinary levels of seven phthalate metabolites in the U.S. population from the national health and nutrition examination survey (NHANES) 1999–2000. *Environ Health Perspect.* 2004;112(3):331–8.
19. Chan M, Mita C, Bellavia A, Parker M, James-Todd T. Racial/Ethnic disparities in pregnancy and prenatal exposure to endocrine-disrupting chemicals commonly used in personal care products. *Curr Environ Health Rep.* 2021;8(2):98–112.
20. Ruiz D, Becerra M, Jagai JS, Ard K, Sargis RM. Disparities in environmental exposures to endocrine-disrupting chemicals and diabetes risk in vulnerable populations. *Diabetes Care.* 2018;41(1):193–205.
21. Branch F, Woodruff TJ, Mitro SD, Zota AR. Vaginal douching and racial/ethnic disparities in phthalates exposures among reproductive-aged women: National health and nutrition examination survey 2001–2004. *Environ Health.* 2015;14:57.
22. Taylor KW, Troester MA, Herring AH, Engel LS, Nichols HB, Sandler DP, et al. Associations between personal care product use patterns and breast cancer risk among white and black women in the sister study. *Environ Health Perspect.* 2018;126(2):027011.
23. Agarwal A, Desai NR, Makker K, Varghese A, Mouradi R, Sabanegh E, et al. Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: An in vitro pilot study. *Fertil Steril.* 2009;92(4):1318–25.
24. Fejes I, Závaczki Z, Szöllösi J, Koloszár S, Daru J, Kovács L, et al. Is there a relationship between cell phone use and semen quality? *Arch Androl.* 2005;51(5):385–93.
25. Rezk AY, Abdulqawi K, Mustafa RM, El-Azm A, Al-Inany TM. Fetal and neonatal responses following maternal exposure to mobile phones. *Saudi Med J.* 2008;29(2):218–23.
26. Gul A, Çelebi H, Uğraş S. The effects of microwave emitted by cellular phones on ovarian follicles in rats. *Arch Gynecol Obstet.* 2009;280(5):729–33.
27. Lyche JL, Rosseland C, Berge G, Polder A. Human health risk associated with brominated flame-retardants (BFRs). *Environ Int.* 2015;74:170–80.
28. Testosterone =98 58-22-0. (n.d.). Retrieved April 9, 2022, from <https://www.sigmaldrich.com/US/en/product/sigma/t1500>.
29. Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, et al. Bisphenol a interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Mol Cell Endocrinol.* 1998 Jul;142(1–2):203–14.
30. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology.* 1998 Oct;139(10):4252–63.
31. Qian Y, Shao H, Ying X, Huang W, Hua Y. The endocrine disruption of prenatal phthalate exposure in mother and offspring. *Front Public Heal.* 2020 Aug 28;8:366.
32. Hannon PR, Flaws JA. The effects of phthalates on the ovary. *Front Endocrinol (Lausanne).* 2015 Feb 2;6:8.
33. Dobrzyńska MM. Phthalates – widespread occurrence and the effect on male gametes. Part 2. The effects of phthalates on male gametes and on the offspring. *Rocznik Panstw Zakl Hig.* 2016;67(3):209–21.
34. Hlisníková H, Petrovičová I, Kolena B, Šídlovská M, Sirotník A. Effects and mechanisms of Phthalates' action on reproductive processes and reproductive health: A literature review. *Int J Environ Res Public Health.* 2020 Sep 18;17(18):6811.
35. Brouwer A, Longnecker MP, Birnbaum LS, Coglianese J, Kostyniak P, Moore J, et al. Characterization of potential endocrine-related health effects at low-dose levels of exposure to PCBs. *Environ Health Perspect.* 1999 Aug;107(Suppl 4):639–49.
36. Phelps J. Headliners: Reproductive health: Effects of organochlorine compounds on menstrual cycles. *Environ Health Perspect.* 2005 Jul;113(7):A455.
37. Bretveld RW, Thomas CMG, Scheepers PTJ, Zielhuis GA, Roeleveld N. Pesticide exposure: The hormonal function of the female reproductive system disrupted? *Reprod Biol Endocrinol.* 2006 May 31;4:30.
38. Zlatnik MG. Endocrine-disrupting chemicals and reproductive health. *J Midwifery Womens Health.* 2016;61(4):442–55.
39. Louis GMB, Chen Z, Schisterman EF, Kim S, Sweeney AM, Sundaram R, et al. Perfluorochemicals and human semen quality: The LIFE study. *Environ Health Perspect.* 2015 Jan;123(1):57–63.
40. Wirth JJ, Mijal RS. Adverse effects of low level heavy metal exposure on male reproductive function. *Syst Biol Reprod Med.* 2010;56(2):147–67.

41. Rzymski P, Tomczyk K, Poniedziałek B, Opala T, Wilczak M. Impact of heavy metals on the female reproductive system. *Ann Agric Environ Med.* 2015;22(2):259–64.
42. Ermeler S, Kortenkamp A. Declining semen quality and polybrominated diphenyl ethers (PBDEs): Review of the literature to support the derivation of a reference dose for a mixture risk assessment. *Int J Hyg Environ Health.* 2022;242:113953.
43. Harley KG, Marks AR, Chevrier J, Bradman A, Sjödin A, Eskenazi B. PBDE concentrations in women's serum and fecundability. *Environ Health Perspect.* 2010 May;118(5):699–704.
44. Bruner-Tran KL, Osteen KG. Dioxin-like PCBs and endometriosis. *Syst Biol Reprod Med.* 2010;56(2):132–46.
45. Sofo V, Götte M, Laganà AS, Salmeri FM, Triolo O, Sturlese E, et al. Correlation between dioxin and endometriosis: An epigenetic route to unravel the pathogenesis of the disease. *Arch Gynecol Obstet.* 2015;292(5):973–86.
46. Costa LG, Giordano G, Tagliaferri S, Cagliari A, Mutti A. Polybrominated diphenyl ether (PBDE) flame retardants: Environmental contamination, human body burden and potential adverse health effects. *Acta Biomed.* 2008;79(3):172–83.
47. Chapin RE, Adams J, Boekelheide K, Gray LEJ, Hayward SW, Lees PSJ, et al. NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A. *Birth Defects Res B Dev Reprod Toxicol.* 2008 Jun;83(3):157–395.
48. Völkel W, Colnot T, Csanády GA, Filser JG, Dekant W. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. *Chem Res Toxicol.* 2002;15(10):1281–7.
49. Genuis SJ, Beeson S, Lobo RA, Birkholz D. Human elimination of phthalate compounds: Blood, urine, and sweat (BUS) study. *ScientificWorldJournal.* 2012;2012:615068.
50. Centers for Disease Control and Prevention. (2017, April 7). Parabens factsheet. Centers for Disease Control and Prevention. Retrieved April 9, 2022, from https://www.cdc.gov/biomonitoring/Parabens_FactSheet.html#:~:text=CDC%20scientists%20found%20Methylparaben%20and,paraben%20than%20non%2DHispanic%20whites.
51. Polychlorinated Biphenyls (PCBs). Polychlorinated biphenyls (PCBs). (n.d.). Retrieved April 9, 2022, from [http://www.idph.state.il.us/envhealth/factsheets/polychlorinatedbiphenyls.htm#:~:text=Polychlorinated%20biphenyls%20\(PCBs\)%20are%20a,equipment%20like%20capacitors%20and%20transformers.](http://www.idph.state.il.us/envhealth/factsheets/polychlorinatedbiphenyls.htm#:~:text=Polychlorinated%20biphenyls%20(PCBs)%20are%20a,equipment%20like%20capacitors%20and%20transformers.)
52. ATSDR (U.S. Department of Health and Human Services). Chemical and physical information dioxin. Agency Toxic Subst Dis Regist. 1997;(Dd):3–7. chrome-extension://efaidnbmnnibpcajpcgclclefindmkaj/viewer.html?pdfurl=https%3A%2F%2Fwww.atsdr.cdc.gov%2Ftoxprofiles%2Ftp17-c4.pdf&clen=1222723&chunk=true.
53. ASTDR. 3. Chemical and Physical Information 3.1. Identity. 2014;3–7. chrome-extension://efaidnbmnnibpcajpcgclclefindmkaj/viewer.html?pdfurl=https%3A%2F%2Fwww.atsdr.cdc.gov%2Ftoxprofiles%2Ftp104-c3.pdf&clen=1339752&chunk=true.
54. Wong HL, Giesy JP, Siu WHL, Lam PKS. Estrogenic and dioxin-like activities and cytotoxicity of sediments and biota from Hong Kong mudflats. *Arch Environ Contam Toxicol.* 2005;48(4):575–86.
55. Oanh NTP, Kido T, Honma S, Oyama Y, Anh LT, Phuc HD, et al. Androgen disruption by dioxin exposure in 5-year-old Vietnamese children: Decrease in serum testosterone level. *Sci Total Environ.* 2018;640–641:466–74.
56. Perfluorohexanoic acid - sigma-aldrich. (n.d.). Retrieved April 10, 2022, from <https://www.sigmaaldrich.com/US/en/product/sial/43809.>
57. Cheremisinoff NP. Perfluorinated Chemicals (PFCS). Perfluorinated Chem. 2016; chrome-extension://efaidnbmnnibpcajpcgclclefindmkaj/viewer.html?pdfurl=https%3A%2F%2Fwww.atsdr.cdc.gov%2Fsites%2Fplease%2Fdocuments%2Fperfluorinated_chemicals_508.pdf&clen=1119153&chunk=true.
58. Perfluoroalkyl and Polyfluoroalkyl Substances (pfass). Perfluoroalkyl and Polyfluoroalkyl Substances (PFASs) - Enviro Wiki. (n.d.). Retrieved April 9, 2022, from https://www.enviro.wiki/index.php?title=Perfluoroalkyl_and_Polyfluoroalkyl_Substances_%28PFASs%29.
59. Mishra GD, Cooper R, Kuh D. A life course approach to reproductive health: Theory and methods. *Maturitas.* 2010;65(2):92–7.
60. Griswold MD. Spermatogenesis: The commitment to meiosis. *Physiol Rev.* 2016;96(1):1–17.
61. Baerwald AR, Adams GP, Pierson RA. Characterization of ovarian follicular wave dynamics in women. *Biol Reprod.* 2003;69(3):1023–31.
62. Gougeon A. Dynamics of follicular growth in the human: A model from preliminary results. *Hum Reprod.* 1986;1(2):81–7.
63. Velazquez MA, Fleming TP, Watkins AJ. Periconceptional environment and the developmental origins of disease. *J Endocrinol.* 2019;242(1):T33–T49.
64. Hunt PA, Lawson C, Gieske M, Murdoch B, Smith H, Marre A, et al. Bisphenol A alters early oogenesis and follicle formation in the fetal ovary of the rhesus monkey. *Proc Natl Acad Sci USA.* 2012;109(43):17525–30.
65. Ho S-M, Cheong A, Adgent MA, Vevers J, Suen AA, Tam NN, et al. Environmental factors, epigenetics, and developmental origin of reproductive disorders. *Reprod Toxicol.* 2017;68:85–104.
66. Herbst AL, Ulfelder H, Poskanzer DG. Adenocarcinoma of the vagina. *Problems of Birth Defects.* Springer, pp. 217–20, 1971.
67. Huo D, Anderson D, Palmer JR, Herbst AL. Incidence rates and risks of diethylstilbestrol-related clear-cell adenocarcinoma of the vagina and cervix: Update after 40-year follow-up. *Gynecol Oncol.* 2017;146(3):566–71.
68. Brehm E, Flaws JA. Transgenerational effects of endocrine-disrupting chemicals on male and female reproduction. *Endocrinol.* 2019;160(6):1421–35.
69. Zhang Y, Mustieles V, Williams PL, Wylie BJ, Souter I, Calafat AM, et al. Parental preconception exposure to phenol and phthalate mixtures and the risk of preterm birth. *Environ Int.* 2021;151:106440.
70. Maurice C, Dalvai M, Lambrot R, Deschênes A, Scott-Boyer M-P, McGraw S, et al. Early-life exposure to environmental contaminants perturbs the sperm epigenome and induces negative pregnancy outcomes for three generations via the paternal lineage. *Epigenomes.* 2021;5(2):10.
71. Marcho C, Oluwayiose OA, Pilsner JR. The preconception environment and sperm epigenetics. *Andrology.* 2020;8(4):924–42.
72. Yland JJ, Zhang Y, Williams PL, Mustieles V, Vagios S, Souter I, et al. Phthalate and DINCH urinary concentrations across pregnancy and risk of preterm birth. *Environ Pollut.* 2022;292(Pt B):118476.
73. Ferguson KK, Chen YH, VanderWeele TJ, McElrath TF, Meeker JD, Mukherjee B. Mediation of the relationship between maternal phthalate exposure and preterm birth by oxidative stress with repeated measurements across pregnancy. *Environ Health Perspect.* 2017;125(3):488–94.
74. Ferguson KK, Rosen EM, Barrett ES, Nguyen RHN, Bush N, McElrath TF, et al. Joint impact of phthalate exposure and stressful life events in pregnancy on preterm birth. *Environ Int.* 2019;133 (Pt B):105254.
75. Ferguson KK, Rosen EM, Rosario Z, Feric Z, Calafat AM, McElrath TF, et al. Environmental phthalate exposure and preterm birth in the PROTECT birth cohort. *Environ Int.* 2019;132:105099.
76. Ferguson KK, Chin HB. Environmental chemicals and preterm birth: Biological mechanisms and the state of the science. *Curr Epidemiol Rep.* 2017;4(1):56–71.
77. Carson R. Silent Spring. Boston, MA: Houghton Mifflin, 1962.
78. Mills LJ, Chichester C. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Sci Total Environ.* 2005;343(1–3):1–34.
79. Propper CR. The study of endocrine-disrupting compounds: Past approaches and new directions. *Integr Comp Biol.* 2005;45(1):194–200.

80. Schug TT, Johnson AF, Birnbaum LS, Colborn T, Guillette LJ Jr, Crews DP, et al. Minireview: Endocrine disruptors: Past lessons and future directions. *Mol Endocrinol.* 2016 Aug;30(8):833–47.
81. Zoeller RT, Brown TR, Doan LL, Gore AC, Skakkebaek NE, Soto AM, et al. Endocrine-disrupting chemicals and public health protection: A statement of principles from the endocrine society. *Endocrinology.* 2012 Sep;153(9):4097–110.
82. De Coster S, van Larebeke N. Endocrine-disrupting chemicals: Associated disorders and mechanisms of action. *J Environ Public Health.* 2012;2012:713696. <https://doi.org/10.1155/2012/713696>.
83. Kiyama R, Wada-Kiyama Y. Estrogenic endocrine disruptors: Molecular mechanisms of action. *Environ Int.* 2015;83:11–40.
84. Lauretta R, Sansone A, Sansone M, Romanelli F, Appetecchia M. Endocrine disrupting chemicals: Effects on endocrine glands. *Front Endocrinol (Lausanne).* 2019;10:178.
85. Brevini TAL, Zanetto SB, Cillo F. Effects of endocrine disruptors on developmental and reproductive functions. *Curr Drug Targets Immune Endocr Metab Disord.* 2005;5(1):1–10.
86. Zhao Y, Chen J, Wang X, Song Q, Xu H-H, Zhang Y-H. Third trimester phthalate exposure is associated with DNA methylation of growth-related genes in human placenta. *Sci Rep.* 2016 Sep 22;6:33449.
87. Chen C-H, Jiang SS, Chang I-S, Wen H-J, Sun C-W, Wang S-L. Association between fetal exposure to phthalate endocrine disruptor and genome-wide DNA methylation at birth. *Environ Res.* 2018;162:261–70.
88. Lu X, Fraszczak E, van der Meer TP, van Faassen M, Bloks VW, Kema IP, et al. An epigenome-wide association study identifies multiple DNA methylation markers of exposure to endocrine disruptors. *Environ Int.* 2020;144:106016.
89. Calaf GM, Ponce-Cusi R, Aguayo F, Muñoz JP, Bleak TC. Endocrine disruptors from the environment affecting breast cancer. *Oncol Lett.* 2020;20(1):19–32.
90. Hill B, Skouteris H, Boyle JA, Bailey C, Walker R, Thangaratinam S, et al. Health in preconception, pregnancy and postpartum global Alliance: International network pregnancy priorities for the prevention of maternal obesity and related pregnancy and long-term complications. *J Clin Med.* 2020 Mar 18;9(3):822.
91. Banerjee BD, Koner BC, Ray A. Influence of stress on DDT-induced humoral immune responsiveness in mice. *Environ Res.* 1997;74(1):43–7.
92. Staples JE, Fiore NC, Frazier DEJ, Gasiewicz TA, Silverstone AE. Overexpression of the anti-apoptotic oncogene, bcl-2, in the thymus does not prevent thymic atrophy induced by estradiol or 2,3,7, 8-tetrachlorodibenzo-p-dioxin. *Toxicol Appl Pharmacol.* 1998;151(1):200–10.
93. Chapin RE, Harris MW, Davis BJ, Ward SM, Wilson RE, Mauney MA, et al. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam Appl Toxicol.* 1997 Nov;40(1):138–57.
94. Jin Y, Chen R, Liu W, Fu Z. Effect of endocrine disrupting chemicals on the transcription of genes related to the innate immune system in the early developmental stage of zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* 2010;28(5–6):854–61.
95. Rogers JA, Metz L, Yong VW. Review: Endocrine disrupting chemicals and immune responses: A focus on bisphenol-A and its potential mechanisms. *Mol Immunol.* 2013;53(4):421–30.
96. Henley DV, Korach KS. Endocrine-disrupting chemicals use distinct mechanisms of action to modulate endocrine system function. *Endocrinology.* 2006;147(6):25–32.
97. Atrash HK, Johnson K, Adams M, Cordero JF, Howse J. Preconception care for improving perinatal outcomes: The time to act. *Matern Child Health J.* 2006 Sep;10(5 Suppl):S3–11.
98. Walker CL. Minireview: Epigenomic plasticity and vulnerability to EDC exposures. *Mol Endocrinol.* 2016;30(8):848–55. <https://doi.org/10.1210/me.2016-1086>.
99. Costa LG, de Laat R, Tagliaferri S, Pellacani C. A mechanistic view of polybrominated diphenyl ether (PBDE) developmental neurotoxicity. *Toxicol Lett.* 2014 Oct 15;230(2):282–94.
100. Jayaraj R, Megha P, Sreedev P. Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment. *Interdiscip Toxicol.* 2016 Dec;9(3–4):90–100.
101. Gaskins AJ, Mínguez-Alarcón L, Fong KC, Abdelmessih S, Coull BA, Chavarro JE, et al. Exposure to fine particulate matter and ovarian reserve among women from a fertility clinic. *Epidemiology.* 2019;30(4):486.
102. Gaskins AJ, Mínguez-Alarcón L, VoPham T, Hart JE, Chavarro JE, Schwartz J, et al. Impact of ambient temperature on ovarian reserve. *Fertil Steril.* 2021;116(4):1052–60.
103. Feng X, Luo J, Wang X, Xie W, Jiao J, Wu X, et al. Association of exposure to ambient air pollution with ovarian reserve among women in Shanxi province of north China. *Environ Pollut.* 2021;278:116868.
104. Mahalingaiah S, Hart J, Laden F, Farland L, Hewlett M, Chavarro J, et al. Adult air pollution exposure and risk of infertility in the Nurses' health study II. *Hum Reprod.* 2016;31(3):638–47.
105. Slama R, Bottagisi S, Solansky I, Lepeule J, Giorgis-Allemand L, Sram R. Short-term impact of atmospheric pollution on fecundability. *Epidemiology.* 2013;24(6):871–9.
106. Li Q, Zheng D, Wang Y, Li R, Wu H, Xu S, et al. Association between exposure to airborne particulate matter less than 2.5 μ m and human fecundity in China. *Environ Int.* 2021;146:106231.
107. Messerlian C, Souter I, Gaskins AJ, Williams PL, Ford JB, Chiu Y-H, et al. Urinary phthalate metabolites and ovarian reserve among women seeking infertility care. *Hum Reprod.* 2016;31(1):75–83.
108. Cao M, Pan W, Shen X, Li C, Zhou J, Liu J. Urinary levels of phthalate metabolites in women associated with risk of premature ovarian failure and reproductive hormones. *Chemosphere.* 2020;242:125206.
109. Specht IO, Bonde JP, Toft G, Lindh CH, Jönsson BA, Jørgensen KT. Serum phthalate levels and time to pregnancy in couples from Greenland, Poland and Ukraine. *PloS One.* 2015;10(3):e0120070.
110. Louis GMB, Sundaram R, Sweeney AM, Schisterman EF, Maisog J, Kannan K. Urinary bisphenol A, phthalates, and couple fecundity: The longitudinal investigation of fertility and The environment (LIFE) study. *Fertil Steril.* 2014;101(5):1359–66.
111. Burdorf A, Brand T, Jaddoe V, Hofman A, Mackenbach J, Steegers E. The effects of work-related maternal risk factors on time to pregnancy, preterm birth and birth weight: The generation r study. *Occup Environ Med.* 2011;68(3):197–204.
112. Hougaard KS, Hannerz H, Feveile H, Bonde JP. Increased incidence of infertility treatment among women working in the plastics industry. *Reprod Toxicol.* 2009;27(2):186–9.
113. Ziv-Gal A, Flaws JA. Evidence for bisphenol a-induced female infertility: A review (2007–2016). *Fertil Steril.* 2016;106(4):827–56.
114. Vélez MP, Arbuckle TE, Fraser WD. Female exposure to phenols and phthalates and time to pregnancy: The maternal-infant research on environmental chemicals (MIREC) study. *Fertil Steril.* 2015;103(4):1011–20.
115. Smarr MM, Sundaram R, Honda M, Kannan K, Louis GMB. Urinary concentrations of parabens and other antimicrobial chemicals and their association with couples' fecundity. *Environ Health Perspect.* 2017;125(4):730–6.
116. Yang C-Y, Wang Y-J, Chen P-C, Tsai S-J, Guo YL. Exposure to a mixture of polychlorinated biphenyls and polychlorinated dibenzofurans resulted in a prolonged time to pregnancy in women. *Environ Health Perspect.* 2008;116(5):599–604.
117. Law DCG, Klebanoff MA, Brock JW, Dunson DB, Longnecker MP. Maternal serum levels of polychlorinated biphenyls and 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene (DDE) and time to pregnancy. *Am J Epidemiol.* 2005;162(6):523–32.

118. Buck Louis G, Dmochowski J, Lynch C, Kostyniak P, McGuinness B, Vena J. Polychlorinated biphenyl serum concentrations, lifestyle and time-to-pregnancy. *Hum Reprod.* 2009;24(2):451–8.
119. Fei C, McLaughlin JK, Lipworth L, Olsen J. Maternal levels of perfluorinated chemicals and subfecundity. *Hum Reprod.* 2009;24(5):1200–5.
120. Whitworth KW, Haug LS, Baird DD, Becher G, Hoppin JA, Skjaerven R, et al. Perfluorinated compounds and subfecundity in pregnant women. *Epidemiology.* 2012;23(2):257.
121. Jørgensen KT, Specht IO, Lenters V, Bach CC, Rylander L, Jönsson BA, et al. Perfluoroalkyl substances and time to pregnancy in couples from Greenland, Poland and Ukraine. *Environ Health.* 2014;13(1):1–8.
122. Louis GMB, Sundaram R, Schisterman EF, Sweeney AM, Lynch CD, Gore-Langton RE, et al. Persistent environmental pollutants and couple fecundity: The LIFE study. *Environ Health Perspect.* 2013;121(2):231–6.
123. Louis GMB, Sundaram R, Schisterman EF, Sweeney AM, Lynch CD, Gore-Langton RE, et al. Heavy metals and couple fecundity, the LIFE study. *Chemosphere.* 2012;87(11):1201–7.
124. Bloom MS, Louis GMB, Sundaram R, Kostyniak PJ, Jain J. Associations between blood metals and fecundity among women residing in New York state. *Reprod Toxicol.* 2011;31(2):158–63.
125. Levine H, Jørgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, et al. Temporal trends in sperm count: A systematic review and meta-regression analysis. *Hum Reprod Update.* 2017;23(6):646–59.
126. Jørgensen N, Lamb DJ, Levine H, Pastuszak AW, Sigalos JT, Swan SH, et al. Are worldwide sperm counts declining? *Fertility and Sterility.* 2021;116(6):1457–63.
127. Jurewicz J, Dziewirska E, Radwan M, Hanke W. Air pollution from natural and anthropic sources and male fertility. *Reprod Biol Endocrinol.* 2018;16(1):1–18.
128. Thonneau P, Bujan L, Multigner L, Mieusset R. Occupational heat exposure and male fertility: A review. *Hum Reprod.* 1998;13(8):2122–5.
129. Durairajayagam D, Agarwal A, Ong C. Causes, effects and molecular mechanisms of testicular heat stress. *Reprod Biomed Online.* 2015;30(1):14–27.
130. Jung A, Eberl M, Schill W. Improvement of semen quality by nocturnal scrotal cooling and moderate behavioural change to reduce genital heat stress in men with oligoasthenoteratozoospermia. *Reproduction.* 2001;121(4):595–603.
131. Mínguez-Alarcón L, Gaskins AJ, Chiu Y-H, Messerlian C, Williams PL, Ford JB, et al. Type of underwear worn and markers of testicular function among men attending a fertility center. *Hum Reprod.* 2018;33(9):1749–56.
132. Rodprasert W, Toppari J, Virtanen HE. Endocrine disrupting chemicals and reproductive health in boys and men. *Front Endocrinol.* 2021;12:706532.
133. Meeker JD, Yang T, Ye X, Calafat AM, Hauser R. Urinary concentrations of parabens and serum hormone levels, semen quality parameters, and sperm DNA damage. *Environ Health Perspect.* 2011;119(2):252–7.
134. Chiu Y, Afeiche M, Gaskins A, Williams P, Petrozza J, Tanrikut C, et al. Fruit and vegetable intake and their pesticide residues in relation to semen quality among men from a fertility clinic. *Hum Reprod.* 2015;30(6):1342–51.
135. Hauser R (ed.). The environment and male fertility: Recent research on emerging chemicals and semen quality. Seminars in Reproductive Medicine, New York. Thieme Medical Publishers, Inc., 2006.
136. Mumford SL, Kim S, Chen Z, Gore-Langton RE, Barr DB, Louis GMB. Persistent organic pollutants and semen quality: The LIFE study. *Chemosphere.* 2015;135:427–35.
137. Tarapore P, Ouyang B. Perfluoroalkyl chemicals and male reproductive health: Do PFOA and PFOS increase risk for male infertility? *Int J Environ Res Public Health.* 2021;18(7):3794.
138. Joensen UN, Bossi R, Leffers H, Jensen AA, Skakkebæk NE, Jørgensen N. Do perfluoroalkyl compounds impair human semen quality? *Environ Health Perspect.* 2009;117(6):923–7.
139. López-Botella A, Velasco I, Acién M, Sáez-Espínosa P, Todolí-Torró J-L, Sánchez-Romero R, et al. Impact of heavy metals on human male fertility—An overview. *Antioxidants.* 2021;10(9):1473.
140. Carré J, Gatimel N, Moreau J, Parinaud J, Léandri R. Does air pollution play a role in infertility?: A systematic review. *Environ Health.* 2017;16(1):1–16.
141. Gaskins AJ, Minguez-Alarcon L, Williams PL, Chavarro JE, Schwartz JD, Kloog I, et al. Ambient air pollution and risk of pregnancy loss among women undergoing assisted reproduction. *Environ Res.* 2020;191:110201.
142. Du Y-Y, Fang Y-L, Wang Y-X, Zeng Q, Guo N, Zhao H, et al. Follicular fluid and urinary concentrations of phthalate metabolites among infertile women and associations with in vitro fertilization parameters. *Reprod Toxicol.* 2016;61:142–50.
143. Wu H, Ashcraft L, Whitcomb BW, Rahil T, Tougas E, Sites CK, et al. Parental contributions to early embryo development: Influences of urinary phthalate and phthalate alternatives among couples undergoing IVF treatment. *Hum Reprod.* 2017;32(1):65–75.
144. Machtinger R, Gaskins AJ, Racowsky C, Mansur A, Adir M, Baccarelli AA, et al. Urinary concentrations of biomarkers of phthalates and phthalate alternatives and IVF outcomes. *Environ Int.* 2018;111:23–31.
145. Mínguez-Alarcón L, Souter I, Chiu Y-H, Williams PL, Ford JB, Ye X, et al. Urinary concentrations of cyclohexane-1, 2-dicarboxylic acid monohydroxy isononyl ester, a metabolite of the non-phthalate plasticizer di (isononyl) cyclohexane-1, 2-dicarboxylate (DINCH), and markers of ovarian response among women attending a fertility center. *Environ Res.* 2016;151:595–600.
146. Bloom MS, Kim D, Vom Saal FS, Taylor JA, Cheng G, Lamb JD, et al. Bisphenol a exposure reduces the estradiol response to gonadotropin stimulation during in vitro fertilization. *Fertil Steril.* 2011;96(3):672–7.e2.
147. Mok-Lin E, Ehrlich S, Williams PL, Petrozza J, Wright DL, Calafat AM, et al. Urinary bisphenol A concentrations and ovarian response among women undergoing IVF. *Int J Androl.* 2010;33(2):385–93.
148. Ehrlich S, Williams PL, Missmer SA, Flaws JA, Berry KF, Calafat AM, et al. Urinary bisphenol A concentrations and implantation failure among women undergoing in vitro fertilization. *Environ Health Perspect.* 2012;120(7):978–83.
149. Ehrlich S, Williams PL, Missmer SA, Flaws JA, Ye X, Calafat AM, et al. Urinary bisphenol A concentrations and early reproductive health outcomes among women undergoing IVF. *Hum Reprod.* 2012;27(12):3583–92.
150. Radwan P, Wielgomas B, Radwan M, Krasiński R, Klimowska A, Zajdel R, et al. Triclosan exposure and in vitro fertilization treatment outcomes in women undergoing in vitro fertilization. *Environ Sci Pollut Res Int.* 2021;28(10):12993–9.
151. Hua R, Zhou Y, Wu B, Huang Z, Zhu Y, Song Y, et al. Urinary triclosan concentrations and early outcomes of in vitro fertilization-embryo transfer. *Reproduction.* 2017;153(3):319–25.
152. Dodge LE, Williams PL, Williams MA, Missmer SA, Toth TL, Calafat AM, et al. Paternal urinary concentrations of parabens and other phenols in relation to reproductive outcomes among couples from a fertility clinic. *Environ Health Perspect.* 2015;123(7):665–71.
153. Meeker JD, Maity A, Missmer SA, Williams PL, Mahalingaiah S, Ehrlich S, et al. Serum concentrations of polychlorinated biphenyls in relation to in vitro fertilization outcomes. *Environ Health Perspect.* 2011;119(7):1010–6.
154. Johnson PI, Altshul L, Cramer DW, Missmer SA, Hauser R, Meeker JD. Serum and follicular fluid concentrations of polybrominated diphenyl ethers and in-vitro fertilization outcome. *Environ Int.* 2012;45:9–14.

155. Ingle ME, Mínguez-Alarcón L, Carignan CC, Stapleton HM, Williams PL, Ford JB, et al. Exploring reproductive associations of serum polybrominated diphenyl ether and hydroxylated brominated diphenyl ether concentrations among women undergoing in vitro fertilization. *Hum Reprod.* 2020;35(5):1199–210.
156. Carignan CC, Mínguez-Alarcón L, Butt CM, Williams PL, Meeker JD, Stapleton HM, et al. Urinary concentrations of organophosphate flame retardant metabolites and pregnancy outcomes among women undergoing in vitro fertilization. *Environ Health Perspect.* 2017;125(8):087018.
157. Messerlian C, Williams PL, Mínguez-Alarcón L, Carignan CC, Ford JB, Butt CM, et al. Organophosphate flame-retardant metabolite concentrations and pregnancy loss among women conceiving with assisted reproductive technology. *Fertil Steril.* 2018;110(6):1137–44.e1.
158. Carignan CC, Mínguez-Alarcón L, Williams PL, Meeker JD, Stapleton HM, Butt CM, et al. Paternal urinary concentrations of organophosphate flame retardant metabolites, fertility measures, and pregnancy outcomes among couples undergoing in vitro fertilization. *Environ Int.* 2018;111:232–8.
159. Governini L, Orvieto R, Guerranti C, Gambera L, De Leo V, Piomboni P. The impact of environmental exposure to perfluorinated compounds on oocyte fertilization capacity. *J Assist Reprod Genet.* 2011;28(5):415–8.
160. McCoy JA, Bangma JT, Reiner JL, Bowden JA, Schnorr J, Slowey M, et al. Associations between perfluorinated alkyl acids in blood and ovarian follicular fluid and ovarian function in women undergoing assisted reproductive treatment. *Sci Total Environ.* 2017;605–606:9–17.
161. Heffernan AL, Cunningham TK, Drage DS, Aylward LL, Thompson K, Vijayasarathy S, et al. Perfluorinated alkyl acids in the serum and follicular fluid of UK women with and without polycystic ovarian syndrome undergoing fertility treatment and associations with hormonal and metabolic parameters. *Int J Hyg Environ Health.* 2018;221(7):1068–75.
162. Jørgensen KT, Specht IO, Lenters V, Bach CC, Rylander L, Jönsson BA, et al. Perfluoroalkyl substances and time to pregnancy in couples from Greenland, Poland and Ukraine. *Environ Health.* 2014;13:116.
163. Kim YR, White N, Bräunig J, Vijayasarathy S, Mueller JF, Knox CL, et al. Per- and poly-fluoroalkyl substances (PFASs) in follicular fluid from women experiencing infertility in Australia. *Environ Res.* 2020;190:109963.
164. Ingle ME, Bloom MS, Parsons PJ, Steuerwald AJ, Kruger P, Fujimoto VY. Associations between IVF outcomes and essential trace elements measured in follicular fluid and urine: A pilot study. *J Assist Reprod Genet.* 2017;34(2):253–61.
165. Bloom MS, Parsons PJ, Steuerwald AJ, Schisterman EF, Browne RW, Kim K, et al. Toxic trace metals and human oocytes during in vitro fertilization (IVF). *Reprod Toxicol.* 2010;29(3):298–305.
166. Wu S, Wang M, Deng Y, Qiu J, Zhang X, Tan J. Associations of toxic and essential trace elements in serum, follicular fluid, and seminal plasma with in vitro fertilization outcomes. *Ecotoxicol Environ Saf.* 2020;204:110965.
167. Hou HY, Wang D, Zou XP, Yang ZH, Li TC, Chen YQ. Does ambient air pollutants increase the risk of fetal loss? A case-control study. *Arch Gynecol Obstet.* 2014;289(2):285–91.
168. Moridi M, Ziae S, Kazemnejad A. Exposure to ambient air pollutants and spontaneous abortion. *J Obstet Gynaecol Res.* 2014;40(3):743–8.
169. Di Ciaula A, Bilancia M. Relationships between mild PM10 and ozone urban air levels and spontaneous abortion: Clues for primary prevention. *Int J Environ Health Res.* 2015;25(6):640–55.
170. Ha S, Sundaram R, Buck Louis GM, Nobles C, Seenii I, Sherman S, et al. Ambient air pollution and the risk of pregnancy loss: A prospective cohort study. *Fertil Steril.* 2018;109(1):148–53.
171. Ghosh R, Causey K, Burkart K, Wozniak S, Cohen A, Brauer M. Ambient and household PM 2.5 pollution and adverse perinatal outcomes: A meta-regression and analysis of attributable global burden for 204 countries and territories. *PLoS Med.* 2021;18(9):e1003718.
172. Toft G, Jönsson BA, Lindh CH, Jensen TK, Hjollund NH, Vestergaard A, et al. Association between pregnancy loss and urinary phthalate levels around the time of conception. *Environ Health Perspect.* 2012;120(3):458–63.
173. Gao H, Zhang YW, Huang K, Yan SQ, Mao LJ, Ge X, et al. Urinary concentrations of phthalate metabolites in early pregnancy associated with clinical pregnancy loss in Chinese women. *Sci Rep.* 2017;7(1):6800.
174. Yi H, Gu H, Zhou T, Chen Y, Wang G, Jin Y, et al. A pilot study on association between phthalate exposure and missed miscarriage. *Eur Rev Med Pharmacol Sci.* 2016;20(9):1894–902.
175. Mu D, Gao F, Fan Z, Shen H, Peng H, Hu J. Levels of phthalate metabolites in urine of pregnant women and risk of clinical pregnancy loss. *Environ Sci Technol.* 2015;49(17):10651–7.
176. Cantonwine D, Meeker JD, Hu H, Sánchez BN, Lamadrid-Figueroa H, Mercado-García A, et al. Bisphenol A exposure in Mexico City and risk of prematurity: A pilot nested case control study. *Environ Health.* 2010;9:62.
177. Zhang Y, Mustieles V, Yland J, Braun JM, Williams PL, Attaman JA, et al. Association of parental preconception exposure to phthalates and phthalate substitutes with preterm birth. *JAMA Netw Open.* 2020;3(4):e202159.
178. Messerlian C, Braun JM, Mínguez-Alarcón L, Williams PL, Ford JB, Mustieles V, et al. Paternal and maternal urinary phthalate metabolite concentrations and birth weight of singletons conceived by subfertile couples. *Environ Int.* 2017;107:55–64.
179. Zhang Y, Mustieles V, Williams PL, Yland J, Souter I, Braun JM, et al. Prenatal urinary concentrations of phenols and risk of preterm birth: Exploring windows of vulnerability. *Fertil Steril.* 2021;116(3):820–32.
180. Huang S, Li J, Xu S, Zhao H, Li Y, Zhou Y, et al. Bisphenol a and bisphenol s exposures during pregnancy and gestational age – a longitudinal study in China. *Chemosphere.* 2019;237:124426.
181. Cantonwine DE, Ferguson KK, Mukherjee B, McElrath TF, Meeker JD. Urinary bisphenol A levels during pregnancy and risk of preterm birth. *Environ Health Perspect.* 2015;123(9):895–901.
182. Tang R, Chen MJ, Ding GD, Chen XJ, Han XM, Zhou K, et al. Associations of prenatal exposure to phenols with birth outcomes. *Environ Pollut.* 2013;178:115–20.
183. Geer LA, Pycke BFG, Waxenbaum J, Sherer DM, Abulafia O, Halden RU. Association of birth outcomes with fetal exposure to parabens, triclosan and triclocarban in an immigrant population in Brooklyn, New York. *J Hazard Mater.* 2017;323(Pt A):177–83.
184. Aung MT, Ferguson KK, Cantonwine DE, McElrath TF, Meeker JD. Preterm birth in relation to the bisphenol A replacement, bisphenol S, and other phenols and parabens. *Environ Res.* 2019;169: 131–8.
185. Aker AM, Ferguson KK, Rosario ZY, Mukherjee B, Alshawabkeh AN, Cordero JF, et al. The associations between prenatal exposure to triclocarban, phenols and parabens with gestational age and birth weight in northern Puerto Rico. *Environ Res.* 2019;169: 41–51.
186. Mustieles V, Zhang Y, Yland J, Braun JM, Williams PL, Wylie BJ, et al. Maternal and paternal preconception exposure to phenols and preterm birth. *Environ Int.* 2020;137:105523.
187. Messerlian C, Mustieles V, Mínguez-Alarcon L, Ford JB, Calafat AM, Souter I, et al. Preconception and prenatal urinary concentrations of phenols and birth size of singleton infants born to mothers and fathers from the environment and reproductive health (EARTH) study. *Environ Int.* 2018;114:60–8.
188. Govarts E, Nieuwenhuijsen M, Schoeters G, Ballester F, Bloemen K, de Boer M, et al. Birth weight and prenatal exposure to polychlorinated biphenyls (PCBs) and dichlorodiphenylchloroethylene (DDE): A meta-analysis within 12 European birth cohorts. *Environ Health Perspect.* 2012;120(2):162–70.

189. Longnecker MP, Klebanoff MA, Brock JW, Guo X. Maternal levels of polychlorinated biphenyls in relation to preterm and small-for-gestational-age birth. *Epidemiology*. 2005;16(5):641–7.
190. Wolff MS, Engel S, Berkowitz G, Teitelbaum S, Siskind J, Barr DB, et al. Prenatal pesticide and PCB exposures and birth outcomes. *Pediatr Res*. 2007;61(2):243–50.
191. Liew Z, Luo J, Nohr EA, Bech BH, Bossi R, Arah OA, et al. Maternal plasma perfluoroalkyl substances and miscarriage: A nested case-control study in the Danish national birth cohort. *Environ Health Perspect*. 2020;128(4):47007.
192. Darrow LA, Howards PP, Winquist A, Steenland K. PFOA and PFOS serum levels and miscarriage risk. *Epidemiology*. 2014;25(4):505–12.
193. Louis GM, Sapra KJ, Barr DB, Lu Z, Sundaram R. Preconception perfluoroalkyl and polyfluoroalkyl substances and incident pregnancy loss, LIFE study. *Reprod Toxicol*. 2016;65:11–7.
194. Lam J, Koustas E, Sutton P, Johnson PI, Atchley DS, Sen S, et al. The navigation guide—evidence-based medicine meets environmental health: Integration of animal and human evidence for PFOA effects on fetal growth. *Environ Health Perspect*. 2014;122(10):1040–51.
195. Koustas E, Lam J, Sutton P, Johnson PI, Atchley DS, Sen S, et al. The navigation guide—evidence-based medicine meets environmental health: Systematic review of nonhuman evidence for PFOA effects on fetal growth. *Environ Health Perspect*. 2014;122(10):1015–27.
196. Johnson PI, Sutton P, Atchley DS, Koustas E, Lam J, Sen S, et al. The navigation guide—evidence-based medicine meets environmental health: Systematic review of human evidence for PFOA effects on fetal growth. *Environ Health Perspect*. 2014;122(10):1028–39.
197. Blake BE, Fenton SE. Early life exposure to per- and polyfluoroalkyl substances (PFAS) and latent health outcomes: A review including the placenta as a target tissue and possible driver of peri- and postnatal effects. *Toxicology*. 2020;443:152565.
198. Meng Q, Inoue K, Ritz B, Olsen J, Liew Z. Prenatal exposure to perfluoroalkyl substances and birth outcomes; an updated analysis from the Danish National Birth Cohort. *Int J Environ Res Public Health*. 2018;15(9):1832.
199. Wikström S, Lin P-I, Lindh CH, Shu H, Bornehag C-G. Maternal serum levels of perfluoroalkyl substances in early pregnancy and offspring birth weight. *Pediatr Res*. 2020;87(6):1093.
200. Manzano-Salgado CB, Casas M, Lopez-Espinosa M-J, Ballester F, Iñiguez C, Martinez D, et al. Prenatal exposure to perfluoroalkyl substances and birth outcomes in a Spanish birth cohort. *Environ Int*. 2017;108:278–84.
201. Maisonet M, Terrell ML, McGeehin MA, Christensen KY, Holmes A, Calafat AM, et al. Maternal concentrations of polyfluoroalkyl compounds during pregnancy and fetal and postnatal growth in British girls. *Environ Health Perspect*. 2012;120(10):1432–7.
202. Marks KJ, Cutler AJ, Jedd Z, Northstone K, Kato K, Hartman TJ. Maternal serum concentrations of perfluoroalkyl substances and birth size in British boys. *Int J Hyg Environ Health*. 2019;222(5):889–95.
203. Apelberg BJ, Witter FR, Herbstman JB, Calafat AM, Halden RU, Needham LL, et al. Cord serum concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in relation to weight and size at birth. *Environ Health Perspect*. 2007;115(11):1670–6.
204. Sagiv SK, Rifas-Shiman SL, Fleisch AF, Webster TF, Calafat AM, Ye X, et al. Early-pregnancy plasma concentrations of perfluoroalkyl substances and birth outcomes in project viva: Confounded by pregnancy hemodynamics? *Am J Epidemiol*. 2018;187(4):793–802.
205. Darrow LA, Stein CR, Steenland K. Serum perfluorooctanoic acid and perfluorooctane sulfonate concentrations in relation to birth outcomes in the mid-Ohio valley, 2005–2010. *Environ Health Perspect*. 2013;121(10):1207–13.
206. Starling AP, Adgate JL, Hamman RF, Kechris K, Calafat AM, Ye X, et al. Perfluoroalkyl substances during pregnancy and offspring weight and adiposity at birth: Examining mediation by maternal fasting glucose in the healthy start study. *Environ Health Perspect*. 2017;125(6):067016.
207. Xu C, Yin S, Liu Y, Chen F, Zhong Z, Li F, et al. Prenatal exposure to chlorinated polyfluoroalkyl ether sulfonic acids and perfluoroalkyl acids: Potential role of maternal determinants and associations with birth outcomes. *J Hazard Mater*. 2019;380:120867.
208. Liu X, Chen D, Wang B, Xu F, Pang Y, Zhang L, et al. Does low maternal exposure to per- and polyfluoroalkyl substances elevate the risk of spontaneous preterm birth? A nested case-control study in China. *Environ Sci Technol*. 2020;54(13):8259–68.
209. Khanam R, Kumar I, Oladapo-Shittu O, Twose C, Islam AA, Biswal SS, et al. Prenatal environmental metal exposure and preterm birth: A scoping review. *Int J Environ Res Public Health*. 2021;18(2).
210. Zhu M, Fitzgerald EF, Gelberg KH, Lin S, Druschel CM. Maternal low-level lead exposure and fetal growth. *Environ Health Perspect*. 2010;118(10):1471–5.
211. Rahman A, Vahter M, Smith AH, Nermell B, Yunus M, El Arifeen S, et al. Arsenic exposure during pregnancy and size at birth: A prospective cohort study in Bangladesh. *Am J Epidemiol*. 2009;169(3):304–12.
212. Al-Saleh I, Shinwari N, Mashhour A, Rabah A. Birth outcome measures and maternal exposure to heavy metals (lead, cadmium and mercury) in Saudi Arabian population. *Int J Hyg Environ Health*. 2014;217(2-3):205–18.
213. Rhee JW, Aks SE. Chapter 81 - Organochlorine Insecticides. In: Haddad and Winchester's Clinical Management of Poisoning and Drug Overdose, 4th edition, Shannon MW, Borron SW, Burns MJ (eds.). Philadelphia: W.B. Saunders, pp. 1231–6, 2007. Available from: <https://www.sciencedirect.com/science/article/pii/B9780721606934500864>
214. Harper JC, Hammarberg K, Simopoulou M, Koert E, Pedro J, Massin N, et al. The international fertility education initiative: Research and action to improve fertility awareness. *Hum Reprod Open*. 2021;2021(4):hoab031. <https://doi.org/10.1093/hropen/hoab031>.
215. Harville EW, Mishra GD, Yeung E, Mumford SL, Schisterman EF, Jukic AM, et al. The preconception period analysis of risks and exposures influencing health and development (PrePARED) consortium. *Paediatr Perinat Epidemiol*. 2019 Nov;33(6):490–502.
216. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: A systematic review and meta-analysis. *Hum Reprod*. 2012 Oct;27(10):2908–17.
217. Oluwayiose OA, Marcho C, Wu H, Houle E, Krawetz SA, Suvorov A, et al. Paternal preconception phthalate exposure alters sperm methylome and embryonic programming. *Environ Int*. 2021;155:106693.
218. Eichenlaub-Ritter U, Pacchierotti F. Bisphenol A effects on mammalian oogenesis and epigenetic integrity of oocytes: A case study exploring risks of endocrine disrupting chemicals. *Biomed Res Int*. 2015;2015:698795. <https://doi.org/10.1155/2015/698795>.
219. Santangeli S, Maradonna F, Olivotto I, Piccinetti CC, Gioachini G, Carnevali O. Effects of BPA on female reproductive function: The involvement of epigenetic mechanism. *Gen Comp Endocrinol*. 2017;245:122–6.
220. Hipwell AE, Kahn LG, Factor-Litvak P, Porucznik CA, Siegel EL, Fichorova RN, et al. Exposure to non-persistent chemicals in consumer products and fecundability: A systematic review. *Hum Reprod Update*. 2019 Jan 1;25(1):51–71. <https://doi.org/10.1093/humupd/dmy032>.
221. Pocar P, Fiandanese N, Berrini A, Secchi C, Borromeo V. Maternal exposure to di(2-ethylhexyl)phthalate (DEHP) promotes the transgenerational inheritance of adult-onset reproductive dysfunctions through the female germline in mice. *Toxicol Appl Pharmacol*. 2017;322:113–21.

222. Xin F, Susiarjo M, Bartolomei MS. Multigenerational and transgenerational effects of endocrine disrupting chemicals: A role for altered epigenetic regulation? *Semin Cell Dev Biol.* 2015;43: 66–75.
223. Ghosh A, Tripathy A, Ghosh D. Impact of endocrine disrupting chemicals (EDCs) on reproductive health of human. *Proc Zool Soc.* 2022;75(1):16–30. <https://doi.org/10.1007/s12595-021-00412-3>.
224. Joensen UN, Jørgensen N, Thyssen JP, Szecsi PB, Stender S, Petersen JH, et al. Urinary excretion of phenols, parabens and benzophenones in young men: Associations to reproductive hormones and semen quality are modified by mutations in the Filaggrin gene. *Environ Int.* 2018;121:365–74. <https://www.sciencedirect.com/science/article/pii/S0160412018310274>.
225. Braun JM, Messerlian C, Hauser R. Fathers matter: Why it's time to consider the impact of paternal environmental exposures on children's health. *Curr Epidemiol Rep.* 2017;4(1):46–55.
226. Jenkins TG, Carrell DT. The paternal epigenome and embryogenesis: Poising mechanisms for development. *Asian J Androl.* 2011;13(1):76–80.
227. Kumar M, Kumar K, Jain S, Hassan T, Dada R. Novel insights into the genetic and epigenetic paternal contribution to the human embryo. *Clinics (Sao Paulo).* 2013;68(Suppl 1):5–14.
228. Chen Q, Yan W, Duan E. Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. *Nat Rev Genet.* 2016 Dec;17(12):733–43.
229. Smarr MM, Grantz KL, Sundaram R, Maisog JM, Kannan K, Louis GMB. Parental urinary biomarkers of preconception exposure to bisphenol A and phthalates in relation to birth outcomes. *Environ Heal.* 2015;14(1):73. <https://doi.org/10.1186/s12940-015-0060-5>.
230. El Ouazzani H, Fortin S, Venisse N, Dupuis A, Rouillon S, Cambien G, et al. Perinatal environmental health education intervention to reduce exposure to endocrine disruptors: The PREVED project. *Int J Environ Res Public Health.* 2021 Dec 22;19(1):70.
231. Park S, Chung C. Effects of A dietary modification intervention on menstrual pain and urinary BPA levels: A single group clinical trial. *BMC Womens Health.* 2021;21(1):58.
232. Galloway TS, Baglin N, Lee BP, Kocur AL, Shepherd MH, Steele AM, et al. An engaged research study to assess the effect of a “real-world” dietary intervention on urinary bisphenol A (BPA) levels in teenagers. *BMJ Open.* 2018;8(2):e018742.
233. Szybiak A, Rutkowska A, Wilczewska K, Wasik A, Namieśnik J, Rachon D. Daily diet containing canned products significantly increases serum concentrations of endocrine disruptor bisphenol A in young women. *Polish Arch Intern Med.* 2017;127(4): 278–80.
234. Barrett ES, Velez M, Qiu X, Chen S-R. Reducing prenatal phthalate exposure through maternal dietary changes: Results from a pilot study. *Matern Child Health J.* 2015;19(9):1936–42.
235. Carwile JL, Ye X, Zhou X, Calafat AM, Michels KB. Canned soup consumption and urinary bisphenol A: A randomized crossover trial. *JAMA.* 2011;306(20):2218–20.
236. Rudel RA, Gray JM, Engel CL, Rawsthorne TW, Dodson RE, Ackerman JM, et al. Food packaging and bisphenol A and bis(2-ethylhexyl) phthalate exposure: Findings from a dietary intervention. *Environ Health Perspect.* 2011 Jul;119(7):914–20.
237. Hagopian T, Delli-Bovi Z, Mercado A, Bird A, Guy M, Phelan S. Development and feasibility of randomized trial to reduce urinary bisphenols in women with obesity. *Pilot Feasibility Stud.* 2021;7(1):24.
238. Jo A, Kim S, Ji K, Kho Y, Choi K. Influence of vegetarian dietary intervention on urinary paraben concentrations: A pilot study with ‘temple stay’ participants. *Toxics.* 2020 Jan 17;8(1):3.
239. Sears CG, Lanphear BP, Calafat AM, Chen A, Skarha J, Xu Y, et al. Lowering urinary phthalate metabolite concentrations among children by reducing contaminated dust in housing units: A randomized controlled trial and observational study. *Environ Sci Technol.* 2020 Apr 7;54(7):4327–35.

INDICATIONS FOR IN VITRO FERTILIZATION TREATMENT

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Introduction

In vitro fertilization (IVF) was initially developed to treat tubal infertility. The first live birth achieved with IVF was reported in 1978 in a women with a bilateral obstruction of fallopian tubes [1]. This technique of extracorporeal fertilization was the only option to achieve pregnancy after a previous attempt of bilateral salpingostomy had failed. The oocyte was retrieved from a natural ovulatory cycle by laparoscopy and was fertilized *in vitro* before being transferred in utero. Since then, indications of IVF have widened. It is estimated that more than eight million babies have been born using IVF worldwide [2, 3]. According to European registries, 165,379 cycles of IVF were performed in 2017, with a total number of IVF cycles increasing year after year [2, 4]. However, this expansion of IVF practice raises medical and ethical questions. IVF is associated with medical risks for the patient [5–7], as well as to adverse obstetrical, perinatal, and neonatal outcomes compared to spontaneous pregnancies [3, 8–11]. Moreover, the practice of IVF leads to an increasing number of frozen embryos, notably due to freeze-all strategies to prevent ovarian hyperstimulation syndrome, in order to circumvent the issue of elevated progesterone levels in the late follicular phase and to obtain a maximum number of embryos after one single oocyte retrieval [12–14]. This growing number of supernumerary embryos that might not be used by couples raises an ethical question about the future of these embryos and the production of human material. In all, the development of IVF and its increasing success rates has opened a wide range of perspectives, engaging new visions of human society [15]. In light of the evolution of IVF practices questioning the moral status of the human embryo and the ethics of IVF practice, this chapter reviews the indications of IVF treatment, whether commonly admitted or controversial.

Tubal infertility

As already mentioned, IVF was initially developed to treat tubal infertility. The permeability of fallopian tubes is a major parameter in human fertility, as the oocyte is fertilized in the fallopian tubes and the first stages of embryo development occur during its journey from the tubes to the uterine cavity. Tubal obstruction is reported in 12% to 33% of infertile couples [16] and can be diagnosed by hysterosalpingography (a radiographic evaluation of the uterine cavity and fallopian tubes after injection of radio-opaque medium through the cervical canal) [17], by ultrasound scan (hysterosalpingo-contrast sonography [HyCoSy] or hysterosalpingo-foam sonography [HyFoSy]) [18, 19], or by laparoscopy and dye test. The integrity of fallopian tubes and their function can be affected by multiple factors such as tubal obstruction or occlusion (whether proximal, distal, unilateral, or bilateral), peritubal adhesions, pelvic inflammatory disease, endometriosis, or by tubal surgery [20]. Tubal infections can be a consequence of sexually transmitted diseases, post-pregnancy sepsis, intrauterine contraceptive devices, or post-surgery complications. The

most common infection affecting tubes is *Chlamydia trachomatis*, reported to be significantly associated with bilateral tubal obstruction [21]. Other infectious agents such as gonorrhoea may also induce tubal damage, with 30% to 50% of patients with gonococcus having a concomitant infection with *Chlamydia trachomatis* [16]. The severity of tubal infertility due to pelvic infections depends on the number and severity of episodes [16].

Tubal surgery is a therapeutic option to restore the chances of spontaneous pregnancy in case of tubal infertility. However, at a worldwide level, randomized controlled trials (RCTs) comparing the benefits and costs of IVF versus reproductive tubal surgery are lacking. First-line surgical treatment in this context and its outcome are related to the site and extent of tubal damage. In case of proximal occlusion, despite insufficient evidence in favour of tubal catheterization compared to first-line IVF [22], it seems that tubal catheterization should be attempted, notably due to its simplicity of execution [23]. A systematic review and meta-analysis of 27 observational studies analysing 1556 patients undergoing tubal catheterization for proximal tubal obstruction and who attempted to conceive naturally after the procedure reported a pooled clinical pregnancy rate of 27% (95% CI: 25%–30%), a pooled cumulative clinical pregnancy rate of 22.3% (95% CI: 17.8%–27.8%) after six months and of 26.4% (95% CI: 23.0%–30.2%) after 12 months [22]. These results after tubal catheterization are almost comparable to that after IVF, with the advantage of having restored natural fertility. In case of distal occlusion, first-line surgical treatment might be considered but remains a subject of controversy, depending on the type of tubal lesion and the availability of qualified surgeons to perform high-quality tubal surgery. Among other elements that can impact tubal permeability, the management of hydrosalpinges also remains debated. Hydrosalpinges are associated with lower pregnancy rates and with higher risks of ectopic pregnancy, even after surgical repair [24–26]. However, surgically treating hydrosalpinges prior to IVF seems to have a positive effect on the chances of IVF success according to a Cochrane review [27]. Similarly, there is no consensus regarding the optimal treatment in case of unilateral tubal infertility. Although intrauterine insemination (IUI) is theoretically possible, IUI in women with unilateral tubal abnormalities has been associated with decreased live birth rates compared to controls [28, 29]. For these specific cases, some studies suggest that IVF might be a suitable first-line therapeutic option that eliminates the risk of ectopic pregnancy by bypassing the fallopian tubes compared to spontaneous conception [30].

Overall, large trials are warranted to establish the effectiveness of tubal surgery in women with tubal infertility, prior to IVF and/or compared to IVF. Results of tubal surgery and subsequent live birth rates also remain to be adequately assessed in relation to the nature and severity of tubal damage. Future trials should also consider the cost-effectiveness of surgery versus IVF in the treatment of tubal infertility [31]. To date, in the absence of solid

data, it seems that IVF should rapidly be considered for couples with a tubal factor of infertility, and even more so in case of failed surgery, poor-prognosis couples, or presence of other factors of infertility.

Endometriosis

Endometriosis is one of the major reasons for consultation in reproductive units. Women with endometriosis have a reduced monthly fecundity rate (2%–10%) compared with fertile couples [32]. Data to establish clear guidelines for endometriosis-associated infertility lack randomized trials evaluating the efficacy of assisted reproductive techniques (ART) versus no intervention in women with endometriosis [33]. Furthermore, the efficacy of surgery for infertile patients with endometriosis is debated. The best treatment option depends on various factors, including the stage of endometriosis. For stage I/II endometriosis, according to rASRM (revised American Society of Reproductive Medicine score), operative laparoscopy can be offered as a treatment option, as some studies observed improved ongoing pregnancy rates after surgery compared to no intervention [34]. According to European Society of Human Reproduction and Embryology (ESHRE) guidelines, surgery with CO₂ laser vaporization should be preferred, as it is associated with higher cumulative spontaneous pregnancy rates compared to monopolar electrocoagulation [32]. After surgery, a scoring system known as the Endometriosis Fertility Index (EFI) can be used to predict the chances of post-surgical natural pregnancy [35]. The score considers patient-related factors (age, duration of infertility, and history of prior pregnancy) and surgical factors (function of the tubes and ovaries, endometriosis lesions, and total score as extracted from the rASRM staging), generating a score from 0 to 10 correlated to the chances of non-ART pregnancy after surgery, and can therefore be used as a tool to counsel patients on their reproductive options [36]. In this context, the place of IUI is unclear. According to ESHRE guidelines, IUI with ovarian stimulation may be performed in infertile women with rASRM stage I/II endometriosis compared to expectant management [37]. In moderate to severe endometriosis, there is no RCT or meta-analysis to assess whether surgery has a positive impact on pregnancy rates. Studies report pregnancy rates ranging from 30% to 67% after surgery, but are not of high quality and do not distinguish the different types of endometriotic lesions [32]. Moreover, there are conflicting arguments to determine whether removing rectovaginal lesions improves spontaneous pregnancy rates, notably since this kind of aggressive surgery is accompanied by a high rate of complications [38, 39]. Anyhow, the benefit of additional surgeries seems limited. Indeed, a systematic review demonstrated that pregnancy rates were lower after re-operative surgery for endometriosis compared to after the first surgery (22% for repetitive surgery versus 40% after the first surgery) [40]. The management of severe/deeply infiltrating endometriosis is complex and referral to a centre with the required expertise is recommended [41].

Altogether, the benefit of surgery for endometriotic patients is hard to determine and predict. Given the paucity of available data and lack of consensus on the proper management of endometriosis-associated infertility, the decision for no intervention versus surgery versus IVF must be made on symptoms, the presence of complex cysts requiring histological diagnosis, age, ovarian reserve, duration of infertility, male factor infertility, and availability of qualified surgeons. IVF is a major therapeutic option for infertile patients with endometriosis, especially if there are coexisting causes of infertility and/or if other treatments have

failed. A specific IVF protocol cannot be recommended. Both GnRH antagonist and agonist protocols can be offered based on patient and physician preferences, as no difference in pregnancy or live birth rate between protocols has been demonstrated [37]. Concerning future perspectives, factors such as dysregulation of steroidogenesis, oxidative stress, cell cycle progression, inflammation, and angiogenesis in the follicular environment and oocytes are all possible contributors to endometriosis-related infertility. Therefore, treatments targeting these mechanisms could improve IVF outcomes for couples with endometriosis-related infertility [42].

Polycystic ovary syndrome (PCOS)

In case of ovulation disorder in the context of PCOS, several RCTs have shown that treatments with ovulation-inducing agents (such as selective oestrogen receptor modulators, aromatase inhibitors, or gonadotrophins), with or without ovulation trigger, were effective in the absence of additional tubal or spermatic anomaly [43]. Hence, a simple induction of ovulation is the first-line treatment in this context. Cumulative live birth rates reach 60% after one year of ovulation induction [44]. In clinical practice, IVF can be considered for patients with PCOS (having no other cause of infertility) in case of absence of pregnancy after a few cycles of ovulation induction.

Non-severe male infertility

Although intracytoplasmic sperm injection (ICSI) is the treatment of choice for severe male infertility, the treatment for mild or moderate male infertility is debatable. In good-prognosis couples, the first-line treatment of mild male factor infertility is generally IUI with ovarian stimulation, as it is less invasive and burdensome than IVF or ICSI. In clinical practice, if pregnancy is not achieved after three to four IUI, more invasive treatments can be considered. There seems to be no advantage of ICSI over IVF in case of non-severe spermatic alterations. A retrospective study including a total of 21,899 patients undergoing their first IVF cycle, of which 18,962 were conventional IVF and 2937 ICSI, did not observe an advantage of ICSI over IVF on pregnancy rates and live birth rates for patients with mild or moderate oligoasthenozoospermia [45]. Moreover, a retrospective analysis suggested that when sperm morphology is not severely impaired and sperm concentration and motility are normal, conventional IVF resulted in improved blastocyst rate and quality compared to ICSI [46]. Therefore, in the absence of other pejorative fertility parameters, IVF should be considered in case of mild male infertility in the absence of pregnancy after three to four failed cycles of IUI.

Unexplained infertility and advanced maternal age

Unexplained infertility refers to a situation of infertility despite no cause being identified in the limit of current medical knowledge (absence of ovulation disorder, tubal patency, or sperm alteration). Unexplained infertility concerns up to 40% of infertile couples [47]. The choice of treatment for unexplained infertility is made all the more difficult and controversial as there is no medical explanation for the infertility. Hence, for couples with unexplained infertility, multiple factors have to be considered in decision-making, such as duration of infertility, ovarian reserve, and maternal age. As such, the prognosis of unexplained infertility is reported to be worse when the duration of infertility exceeds three years and the female partner is >35 years old [48]. Hence, in the absence of pejorative infertility factors (advanced maternal

age, diminished ovarian reserve, long duration of infertility), performing three to four cycles of IUI with ovarian stimulation may be a suitable first-line option. Studies showed that live birth rates after three to six IUI cycles were similar to those after one to two IVF cycles for good-prognosis patients with unexplained infertility [49, 50]. However, in case of unfavourable couple parameters, IVF should be considered as a first-line treatment strategy [48]. IVF yields to pregnancy three months faster compared with IUI in case of unexplained infertility according to a RCT [51] and outcomes of IVF for unexplained infertility are overall encouraging. ASRM reports a live birth rate of 30.4% in this context [52]. Compared to expectant management, a Cochrane review of couples with unexplained infertility showed that IVF led to higher pregnancy rates (OR: 3.24, 95% CI: 1.07–9.80) [53], as well as to higher live birth rates (OR: 22.0, 95% CI: 2.56–189.38) [54]. No advantage of performing ICSI over IVF has been observed [55]. ICSI might even yield higher cancellation rates compared to conventional IVF [56].

Besides, an increasing number of women aged above 35 years old are treated in reproductive units. Indeed, an increasing number of women postpone childbearing due to personal, educational, or professional reasons. The proportion of first births to women aged 35 years old or more is eight times higher than 30 years ago [57]. For these women, a fast-track to IVF seems beneficial, as age is associated with a decline of both ovarian reserve and oocyte quality. The exhaustion of the ovarian reserve is mainly attributed to the pool of non-growing follicles that progressively decreases in time through mechanisms of recruitment, development into dominant follicle, ovulation, and atresia [58–60]. A mathematical model suggested that the decline of non-growing follicles might be bi-exponential, with an acceleration at the age of 37.5 years old [61–63]. This increased depletion rate could be explained by an accelerated initiation of follicular growth during the premenopausal decade [59] and/or by increased follicular atresia at primordial stages [63]. Therefore, it seems that IVF should be the treatment of choice before the exhaustion of the ovarian reserve makes IVF unfeasible. IVF has proven to be more efficient than IUI in case of advanced age [64–66]. Hence, given the growing trend of women delaying childbearing and the relatively higher rate of infertility among older women, IVF seems to be the most appropriate strategy when feasible.

Conclusions

In all, every female and male parameter should be considered in the choice of the best infertility treatment. Further RCTs and high-quality studies are warranted to establish clear guidelines and indications of IVF according to the different situations. IVF has emerged as one of the most widely adopted and successful treatments, offering the possibility to have children for millions of couples worldwide. As this technology is constantly and quickly evolving, the implications and applications of IVF are expanding. Although IVF practice seems relatively cost-effective and safe, decision-making should not only be based on medical and financial considerations but also include ethical and social issues. The rapid advances in IVF practice open new visions of human society that have yet to be defined.

References

- Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet Lond Engl. 1978 Aug 12;2(8085):366.
- European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE), Wyns C, De Geyter C, Calhaz-Jorge C, Kupka MS, Motrenko T, et al. ART in Europe, 2017: Results generated from European registries by ESHRE. Hum Reprod Open. 2021;2021(3):hoab026.
- Berntsen S, Söderström-Anttila V, Wennerholm U-B, Laivuori H, Loft A, Oldereid NB, et al. The health of children conceived by ART: "The chicken or the egg?" Hum Reprod Update. 2019 Mar 1;25(2):137–58.
- ART Success Rates | CDC [Internet]. 2022 [cited 2022 Mar 4]. Available from: <https://www.cdc.gov/art/artdata/index.html>
- Bodri D, Guillén JJ, Polo A, Trullénque M, Esteve C, Coll O. Complications related to ovarian stimulation and oocyte retrieval in 4052 oocyte donor cycles. Reprod Biomed Online. 2008;17(2):237–43.
- Maxwell KN, Cholst IN, Rosenwaks Z. The incidence of both serious and minor complications in young women undergoing oocyte donation. Fertil Steril. 2008 Dec;90(6):2165–71.
- Klemetti R, Sevón T, Gissler M, Hemminki E. Complications of IVF and ovulation induction. Hum Reprod Oxf Engl. 2005 Dec;20(12):3293–300.
- Farhi A, Reichman B, Boyko V, Hourvitz A, Ron-El R, Lerner-Geva L. Maternal and neonatal health outcomes following assisted reproduction. Reprod Biomed Online. 2013 May;26(5):454–61.
- Giorgione V, Parazzini F, Fesslova V, Cipriani S, Candiani M, Inversetti A, et al. Congenital heart defects in IVF/ICSI pregnancy: Systematic review and meta-analysis. Ultrasound Obstet Gynecol. 2018 Jan;51(1):33–42.
- Pandey S, Shetty A, Hamilton M, Bhattacharya S, Maheshwari A. Obstetric and perinatal outcomes in singleton pregnancies resulting from IVF/ICSI: A systematic review and meta-analysis. Hum Reprod Update. 2012 Oct;18(5):485–503.
- Pinborg A, Wennerholm UB, Romundstad LB, Loft A, Aittomaki K, Söderström-Anttila V, et al. Why do singletons conceived after assisted reproduction technology have adverse perinatal outcome? Systematic review and meta-analysis. Hum Reprod Update. 2013 Apr;19(2):87–104.
- Roque M, Valle M, Guimarães F, Sampaio M, Geber S. Freeze-all policy: Fresh vs. frozen-thawed embryo transfer. Fertil Steril. 2015 May;103(5):1190–3.
- Zhu Q, Chen Q, Wang L, Lu X, Lyu Q, Wang Y, et al. Live birth rates in the first complete IVF cycle among 20 687 women using a freeze-all strategy. Hum Reprod. 2018 May 1;33(5):924–9.
- Racca A, Vanni VS, Somigliana E, Reschini M, Viganò P, Santos-Ribeiro S, et al. Is a freeze-all policy the optimal solution to circumvent the effect of late follicular elevated progesterone? A multicentric matched-control retrospective study analysing cumulative live birth rate in 942 non-elective freeze-all cycles. Hum Reprod. 2021 Aug 18;36(9):2463–72.
- Fishel S. First in vitro fertilization baby—This is how it happened. Fertil Steril. 2018 Jul 1;110(1):5–11.
- Khalaf Y. Tubal subfertility. BMJ. 2003 Sep 13;327(7415):610–3.
- Baramki TA. Hysterosalpingography. Fertil Steril. 2005;83(6):1595–606.
- Lo Monte G, Capobianco G, Piva I, Caserta D, Dessole S, Marci R. Hysterosalpingo contrast sonography (HyCoSy): Let's make the point! Arch Gynecol Obstet. 2015 Jan;291(1):19–30.
- Engels V, Medina M, Antolín E, Ros C, Amaro A, De-Guirior C, et al. Feasibility, tolerability, and safety of hysterosalpingo-foam sonography (hyfosy). Multicenter, prospective Spanish study. J Gynecol Obstet Hum Reprod. 2021 May;50(5):102004.
- K O, Nm G, Ka R, Gw B, An B, Rr O, et al. How members of the society for reproductive endocrinology and infertility and society of reproductive surgeons evaluate, define, and manage hydrosalpinges. Fertil Steril. 2012;97(5):1095–100.e1-2.
- Approbato FC, Approbato MS, Maia MCS, Lima YAR, Barbosa MA, Benetti BBC. Bilateral but not unilateral tubal obstruction is associated with positive chlamydia serology. JBRA Assist Reprod. 2020 Jan 30;24(1):20–3.

22. De Silva PM, Chu JJ, Gallos ID, Vidyasagar AT, Robinson L, Coomarasamy A. Fallopian tube catheterization in the treatment of proximal tubal obstruction: A systematic review and meta-analysis. *Hum Reprod.* 2017 Apr;32(4):836–52.
23. Woolcott R, Petchpud A, O'Donnell P, Stanger J. Differential impact on pregnancy rate of selective salpingography, tubal catheterization and wire-guide recanalization in the treatment of proximal fallopian tube obstruction. *Hum Reprod.* 1995 Jun;10(6):1423–6.
24. Kodaman PH, Arici A, Seli E. Evidence-based diagnosis and management of tubal factor infertility. *Curr Opin Obstet Gynecol.* 2004 Jun;16(3):221–9.
25. Practice Committee of the American Society for Reproductive Medicine. Committee opinion: Role of tubal surgery in the era of assisted reproductive technology. *Fertil Steril.* 2012 Mar;97(3):539–45.
26. Van Voorhis BJ, Mejia RB, Schlaff WD, Hurst BS. Is removal of hydrosalpinges prior to in vitro fertilization the standard of care? *Fertil Steril.* 2019 Apr 1;111(4):652–6.
27. Johnson N, van Voorst S, Sowter MC, Strandell A, Mol BWJ. Surgical treatment for tubal disease in women due to undergo in vitro fertilisation. *Cochrane Database Syst Rev.* 2010 Jan;20(1):CD002125.
28. Cochet T, Gatimel N, Moreau J, Cohade C, Fajau C, Lesourd F, et al. Effect of unilateral tubal abnormalities on the results of intrauterine inseminations. *Reprod Biomed Online.* 2017 Sep;35(3):314–7.
29. Berker B, Şükür YE, Kahraman K, Atabekoğlu CS, Sönmezler M, Özmen B, et al. Impact of unilateral tubal blockage diagnosed by hysterosalpingography on the success rate of treatment with controlled ovarian stimulation and intrauterine insemination. *J Obstet Gynaecol.* 2014 Feb;34(2):127–30.
30. Schippert C, Soergel P, Staboulidou I, Bassler C, Gagalick S, Hillemanns P, et al. The risk of ectopic pregnancy following tubal reconstructive microsurgery and assisted reproductive technology procedures. *Arch Gynecol Obstet.* 2012 Mar;285(3):863–71.
31. Pandian Z, Akande VA, Harrild K, Bhattacharya S. Surgery for tubal infertility. *Cochrane Database Syst Rev.* 2008 Jul 16;(3):CD006415.
32. Fadlalaoui A, Bouquet de la Jolinière J, Feki A. Endometriosis and infertility: How and when to treat? *Front Surg.* 2014;1.
33. Endometriosis guideline [Internet]. [cited 2022 Mar 6]. Available from: <https://www.esore.eu/Guidelines-and-Legal/Guidelines/Endometriosis-guideline>
34. Bafort C, Beebejaun Y, Tomassetti C, Bosteels J, Duffy JM. Laparoscopic surgery for endometriosis. *Cochrane Database Syst Rev.* 2020 Oct 23;10:CD011031.
35. Adamson GD, Pasta DJ. Endometriosis fertility index: The new, validated endometriosis staging system. *Fertil Steril.* 2010 Oct;94(5):1609–15.
36. Tomassetti C, Bafort C, Vanhie A, Meuleman C, Fieuws S, Welkenhuysen M, et al. Estimation of the Endometriosis Fertility Index prior to operative laparoscopy. *Hum Reprod.* 2021 Feb 18; 36(3):636–46.
37. Dunselman GAJ, Vermeulen N, Becker C, Calhaz-Jorge C, D'Hooghe T, De Bie B, et al. ESHRE guideline: Management of women with endometriosis. *Hum Reprod.* 2014;29(3):400–12.
38. Donnez J, Squifflet J. Complications, pregnancy and recurrence in a prospective series of 500 patients operated on by the shaving technique for deep rectovaginal endometriotic nodules. *Hum Reprod Oxf Engl.* 2010 Aug;25(8):1949–58.
39. Stepniewska A, Pomini P, Bruni F, Mereu L, Ruffo G, Ceccaroni M, et al. Laparoscopic treatment of bowel endometriosis in infertile women. *Hum Reprod.* 2009 Jul;24(7):1619–25.
40. Vercellini P, Somigliana E, Dagati R, Barbara G, Abbiati A, Fedele L. The second time around: Reproductive performance after repetitive versus primary surgery for endometriosis. *Fertil Steril.* 2009 Oct;92(4):1253–5.
41. Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, et al. ESHRE guideline for the diagnosis and treatment of endometriosis. *Hum Reprod.* 2005 Oct;20(10):2698–704.
42. Corachán A, Pellicer N, Pellicer A, Ferrero H. Novel therapeutic targets to improve IVF outcomes in endometriosis patients: A review and future prospects. *Hum Reprod Update.* 2021 Aug 20;27(5):923–72.
43. George K, Kamath MS, Nair R, Tharyan P. Ovulation triggers in anovulatory women undergoing ovulation induction. *Cochrane Database Syst Rev.* 2014;(1):CD006900.
44. Huang LN, Tan J, Hitkari J, Dahan MH. Should IVF be used as first-line treatment or as a last resort? A debate presented at the 2013 Canadian fertility and andrology society meeting. *Reprod Biomed Online.* 2015 Feb 1;30(2):128–36.
45. Liu L, Wang H, Li Z, Niu J, Tang R. Obstetric and perinatal outcomes of intracytoplasmic sperm injection versus conventional in vitro fertilization in couples with nonsevere male infertility. *Fertil Steril.* 2020 Oct;114(4):792–800.
46. Stimpfle M, Jancar N, Vrtacnik-Bokal E, Virant-Klun I. Conventional IVF improves blastocyst rate and quality compared to ICSI when used in patients with mild or moderate teratozoospermia. *Syst Biol Reprod Med.* 2019 Dec;65(6):458–64.
47. Ray A, Shah A, Gudi A, Homberg R. Unexplained infertility: An update and review of practice. *Reprod Biomed Online.* 2012 Jun 1;24(6):591–602.
48. Carson SA, Kallen AN. Diagnosis and management of infertility: A review. *JAMA.* 2021 Jul 6;326(1):65–76.
49. Homberg R. IUI is a better alternative than IVF as the first-line treatment of unexplained infertility. *Reprod Biomed Online.* 2021 Dec 27;S1472-6483(21):00615–5.
50. Nandi A, Raja G, White D, Tarek E-T. Intrauterine insemination+controlled ovarian hyperstimulation versus in vitro fertilisation in unexplained infertility: A systematic review and meta-analysis. *Arch Gynecol Obstet.* 2021 Oct 12;305(4):805–24.
51. Reindollar RH, Regan MM, Neumann PJ, Levine B-S, Thornton KL, Alper MM, et al. A randomized clinical trial to evaluate optimal treatment for unexplained infertility: The fast track and standard treatment (FASTT) trial. *Fertil Steril.* 2010 Aug;94(3):888–99.
52. Practice committee of the American society for reproductive medicine. Effectiveness and treatment for unexplained infertility. *Fertil Steril.* 2006 Nov;86(5 Suppl 1):S111–114.
53. Pandian Z, Gibreel A, Bhattacharya S. In vitro fertilisation for unexplained subfertility. *Cochrane Database Syst Rev.* 2015;(11):CD003357.
54. Hughes EG, Beecroft ML, Wilkie V, Burville L, Claman P, Tummon I, et al. A multicentre randomized controlled trial of expectant management versus IVF in women with fallopian tube patency. *Hum Reprod.* 2004 May 1;19(5):1105–9.
55. Wang R, Danhof NA, Tjon-Kon-Fat RI, Eijkemans MJ, Bossuyt PM, Mochtar MH, et al. Interventions for unexplained infertility: A systematic review and network meta-analysis. *Cochrane Database Syst Rev.* 2019 Sep 5;2019(9):CD012692.
56. Song J, Liao T, Fu K, Xu J. ICSI does not improve live birth rates but yields higher cancellation rates than conventional IVF in unexplained infertility. *Front Med.* 2020;7:614118.
57. Matthews TJ, Hamilton BE. Delayed childbearing: More women are having their first child later in life. *NCHS Data Brief.* 2009 Aug;(21):1–8.
58. Block E. Quantitative morphological investigations of the follicular system in women; Variations at different ages. *Acta Anat (Basel).* 1952;14(1–2):108–23.
59. Gougeon A, Ecochard R, Thalabard JC. Age-related changes of the population of human ovarian follicles: Increase in the disappearance rate of non-growing and early-growing follicles in aging women. *Biol Reprod.* 1994 Mar;50(3):653–63.
60. Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: Implications for forecasting menopause. *Hum Reprod.* 1992 Nov;7(10):1342–6.

61. Faddy MJ, Gosden RG. A model conforming the decline in follicle numbers to the age of menopause in women. *Hum Reprod.* 1996 Jul;11(7):1484–6.
62. Faddy MJ. Follicle dynamics during ovarian ageing. *Mol Cell Endocrinol.* 2000 May 25;163(1–2):43–8.
63. Faddy MJ, Gosden RG. A mathematical model of follicle dynamics in the human ovary. *Hum Reprod.* 1995 Apr;10(4):770–5.
64. Tsafrir A, Simon A, Margalioth EJ, Laufer N. What should be the first-line treatment for unexplained infertility in women over 40 years of age - ovulation induction and IUI, or IVF? *Reprod Biomed Online.* 2009;19(Suppl 4):4334.
65. Wiser A, Shalom-Paz E, Reinblatt SL, Son W-Y, Das M, Tulandi T, et al. Ovarian stimulation and intrauterine insemination in women aged 40 years or more. *Reprod Biomed Online.* 2012 Feb;24(2):170–3.
66. Goldman MB, Thornton KL, Ryley D, Alper MM, Fung JL, Hornstein MD, et al. A randomized clinical trial to determine optimal infertility treatment in older couples: The Forty and Over Treatment Trial (FORT-T). *Fertil Steril.* 2014 Jun;101(6):1574–81. e1-2.

INITIAL INVESTIGATION OF THE INFERTILE COUPLE

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Infertility is defined as a failure to conceive after 12 months of unprotected intercourse [1]. The World Health Organization (WHO) and many professional societies, including the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), consider infertility to be a disease of the reproductive system [2, 3].

Infertility affects one in seven couples [4]. In general, after one year of unprotected intercourse 80% of women will conceive. Among the remainder, half of them will conceive in the second year [5]. The National Institute for Health and Care (NICE) and the ASRM recommend starting infertility investigations in those with a possibility of impaired reproductive function as suggested by their history or physical findings, regardless of age. In the absence of any known cause of infertility, evaluation should be offered after 12 months of unprotected intercourse in women under 35 years of age, and after six months in those aged 35 or older. In women over 40, an earlier evaluation and treatment may be warranted [3, 5].

General assessment of the couple

The main purpose of the fertility workup is to find a cause for infertility, particularly those that are amendable to treatment. It includes the identification of infertility-associated medical conditions such as various hormonal or genetic disorders. A fertility workup should involve evaluation of the prognostic value of potential assisted reproductive technology (ART) treatment. The causes of infertility include ovulatory disorders (25%), tubal damage (20%), male factors causing infertility (30%), and uterine or peritoneal disorders (10%) [4–6]. The causes of male infertility include obstruction of the genital tract, testicular failure, varicocele, and genetic or ejaculatory disorders [7]. About a quarter of cases of infertility remain unexplained [6]. Both members of the couple need to be evaluated since in about 40% of cases disorders are found in both the male and female [4, 6].

History-taking is a crucial part of the infertility investigation and should include sexual history, such as frequency and timing of sexual intercourse and questions regarding the possibility of sexual dysfunction. It is also important to identify situations requiring specific care, such as history of genetic disease or consanguinity.

The initial evaluation is also used to counsel patients regarding preconception care and lifestyle modification. *Preconception care* includes assessment of rubella status of the female partner. Vaccination should be offered for non-immune patients [5, 8]. Folic acid and vitamin B12 supplementation are recommended before, during, and after pregnancy, and the dosage should be adjusted according to the patient's specific risk for neural tube defect [9]. Cervical cancer screening is another part of the initial preconception evaluation [5, 8]. *Lifestyle modification* includes avoidance of smoking and use of recreational drugs and minimizing alcohol consumption [8]. Both partners should be advised

that obesity ($BMI \geq 30$) is associated with reduced fertility and maternal and fetal risks. Weight reduction is therefore recommended [5, 10].

Testing for infectious disease should be offered to those undergoing *in vitro* fertilization (IVF) treatment, and should include human immunodeficiency virus (HIV), hepatitis B, and hepatitis C [5]. Screening of the female partner especially before undergoing uterine instrumentation (like hysterosalpingography) for *Chlamydia trachomatis* should be considered [5].

Female investigation

History

In addition to general history-taking for both partners, a gynaecological history is essential. History-taking should include a menstrual history, previous pregnancy history and outcomes, a history of sexually transmitted disease, previous methods of contraception, and previous fertility treatments, including a history of pelvic surgeries. Inquiries should include signs of endometriosis, such as dysmenorrhea, dyspareunia, and cyclic or chronic pelvic pain.

General history should focus on weight and on endocrine diseases that could interfere with gonadal function like thyroid disease, galactorrhoea, or hirsutism. Occupation, environmental exposure to toxins, and drug use should be noted. Family history should include any congenital anomalies, developmental delay, early menopause, or other reproductive problems [11].

Physical examination

General physical examination should include the patient's weight and height, identification of thyroid abnormalities, breast secretion, hirsutism, and other signs of hyperandrogenism. This is followed by pelvic examination focusing on vaginal or cervical abnormalities; uterine size, position, and mobility; and cul-de-sac or adnexal masses. Pelvic ultrasound (US) is complementary to the physical examination.

Diagnostic evaluation

Baseline investigations should be performed to assess ovulatory function, ovarian reserve, uterine abnormalities, and fallopian tube pathology.

Ovulatory function

Regular menstrual cycle, occurring at intervals of 21–35 days [12], is usually indicative of normal ovulation. Still, some degree of variation is normal, depending especially on the woman's age [12].

Irregular cycles, oligomenorrhea, or amenorrhea can all be attributed to ovulatory dysfunction.

- Ovulation was historically assessed by serial basal body temperature (BBT) measurement. Although a biphasic BBT provides presumptive evidence of ovulation, monophasic

or uninterpretable BBTs are also common in ovulatory patients. Moreover, BBT cannot accurately predict timing of ovulation [13]. As a result, this test is not recommended [5, 11].

- Commercially available urinary luteinizing hormone (LH) kits identify the mid-cycle LH surge suggesting the presence of ovulation. Although LH kits help to determine the fertile period, they do not improve the chance of natural conception. It could be useful for couples not having regular sexual intercourse. It indicates the fertile period, but their repetitive use may become expensive and frustrating. Reliability and ease of use may vary among different products, and false-positive LH tests have been estimated to occur in 7% of cases [14].
- Mid-luteal serum progesterone testing is an easy method and is the most commonly used test to confirm ovulation. It should be performed on day 21 of a 28-day cycle or seven days before the commencement of menses. Yet the progesterone concentration fluctuates widely, even among ovulatory women, and may impair interpretation. Values greater than 3.0 ng/mL are presumptive that ovulation has occurred [15].
- Transvaginal ultrasound plays a role in confirming ovulation; however, it is time-consuming and costly. Serial US examinations evaluating follicular growth, appearance of the corpus luteum, pelvic fluid, and luteal-appearing changes in endometrial lining could show indirect signs of ovulation.
- Endometrial biopsy and histological dating have been used to evaluate ovulation. However, these tests lack accuracy and precision and could not distinguish between fertile and infertile women [11]. Their use is limited and they have been abandoned as routine tests [5].

Other hormonal tests

The most common cause of ovulatory dysfunction is polycystic ovary syndrome (PCOS). However, other causes such as obesity, weight gain or loss, strenuous exercise, thyroid dysfunction, or hyperprolactinemia should be investigated and treated. WHO distinguished three types of anovulation: hypogonadotropic hypogonadism (WHO type 1), PCOS (WHO type 2), or ovarian failure (WHO type 3) [5]. Therefore, serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), oestradiol, thyroid stimulating hormone (TSH), and prolactin should be measured.

Women with signs and symptoms of hyperandrogenism require further investigations (serum testosterone, $\delta 4$ -androstenedione, dehydroepiandrosterone-sulphate (DHEA-S) and 17-hydroxyprogesterone) to rule out the presence of late-onset congenital adrenal hyperplasia, Cushing syndrome, or androgen-producing tumours.

Ovarian reserve

Ovarian reserve evaluation is an essential component in the infertility workup. The main goal is to aid in predicting ovarian response to controlled ovarian stimulation. In addition, it helps clinicians to choose the optimal stimulation strategy and to avoid iatrogenic complications, such as ovarian hyperstimulation syndrome. Evaluating the ovarian reserve also facilitates appropriate patient counselling [16].

Ovarian reserve tests offer a quantitative rather than a qualitative evaluation of the ovaries. Their value is limited in the prediction of ongoing pregnancy, both for spontaneous conceptions and those achieved by ART [16]. Age remains the best predictor of pregnancy following *in vitro* fertilization (IVF) [17]. For this reason, withholding IVF purely on the basis of ovarian reserve tests is controversial and considered inappropriate [16].

The main tests for ovarian reserve include day-3 serum follicle-stimulating hormone (FSH) and oestradiol (E2), serum anti-Mullerian hormone (AMH), and antral follicle count (AFC). Other ovarian tests such as serum inhibin B or isolated E2, ovarian volume, ovarian flow measurement, and clomiphene citrate challenge test are not recommended. Their predictive values are considered inferior to the aforementioned ovarian markers [5, 11].

Day 3 serum FSH and E2

FSH is downregulated by E2, and these hormonal markers should be interpreted together. Indeed, elevated E2 could otherwise falsely normalize FSH. Early follicular-phase FSH is an indirect marker of ovarian reserve; yet there is high intra- and inter-cycle variability. Sensitivity of FSH to predict poor ovarian response is better at very high threshold levels [18]. If several values are obtained in the same patient, the highest value is considered to be prognostic. The upper threshold of FSH varies between 8.9 and 25 IU/L [5, 18].

Anti-Mullerian hormone

Anti-Mullerian hormone (AMH) is produced by granulosa cells of primary, preantral, and early antral follicles. Serum concentrations of AMH are gonadotropin-independent, therefore remain relatively consistent during the menstrual cycle and between cycles [19]. Normal values of AMH are described by several normograms. According to NICE, AMH levels greater than 3.5 ng/mL are predictive of a high ovarian response, whereas a level under 0.75 ng/mL is predictive of low response [5]. Overall, lower serum AMH levels (<1 ng/mL) have been associated with poor ovarian responses to stimulation, poor embryo quality, and poor pregnancy outcomes in IVF [11]. A caution should be taken while interpreting AMH levels, since they may be diminished in women using hormonal contraceptives [19], or higher in women with PCOS compared to those without [11]. Measuring ovarian reserve is more sensitive using AMH levels rather than FSH levels, since AMH levels tend to decline before the FSH starts rising [19]. For this reason, AMH has largely replaced basal FSH and E2 level testing as a biomarker of ovarian reserve.

Antral follicle count

Antral follicle count (AFC) has been described as the sum of all follicles 2–10 mm in the largest diameter measured by transvaginal US. AFC should be performed during the early follicular phase of the cycle. It is a direct marker of ovarian reserve. According to NICE, AFC greater than 16 is predictive of a high response to ovarian stimulation, whereas an AFC lower than 4 is predictive of a low response [5].

AFC and AMH are highly correlated [19]. They have comparable performance in the prediction of excessive and poor ovarian response to stimulation [17]. The Bologna criteria for poor ovarian response include at least one abnormal ovarian test: AFC <5–7 follicles or AMH <0.5–1.1 ng/mL [20].

Uterine abnormalities

Intrauterine abnormalities, including endometrial polyps, submucosal myoma, adhesions, or a uterine septum, impair fertility and pregnancy rates in assisted reproduction.

Congenital uterine anomalies are developmental structure pathologies that may affect fertility and pregnancy outcomes. Management of uterine anomalies depends on the type and severity of the anomaly, along with the reproductive and obstetric history. For example, communicating rudimentary uterine horns need to be resected to prevent pregnancy from occurring in the horn. In contrast, operative intervention for a bicornuate or didelphic uterus has limited evidence and carries risks of complications and is not recommended [21]. Hysteroscopic metroplasty for a dysmorphic (T-shaped) uterus is controversial but may have some beneficial effect [22, 23].

Common diagnostic tools for evaluating uterine cavities are described in the following list.

- The first-line diagnostic tool to evaluate uterine cavity is two-dimensional transvaginal US. It is inexpensive, easy to perform, and well-tolerated by patients. Its sensitivity in detecting intrauterine lesions ranges from 56% to 89% [24, 25]. US has less diagnostic value in differentiating submucosal fibroids in the presence of multiple fibroids, synechiae, or uterine malformations.
- Three-dimensional (3D) US further delineates two-dimensional ultrasound findings of the uterine cavity, congenital malformations, or ovarian pathology. Three-dimensional US was found to be as effective as magnetic resonance imaging (MRI) in diagnosing uterine malformations, and in some studies was found to have 100% sensitivity [26]. Compared to MRI, 3D US is safer, cheaper, and more tolerable for the patient [26].
- Hysterosalpingography (HSG) evaluates tubal patency and, to a certain extent, assesses the uterine cavity. However, intrauterine defects detected on HSG could also be due to air bubbles, mucus, or menstrual debris. False-negative findings may be the result of excessive contrast media obliterating shadows caused by small lesions. For evaluation of the uterine cavity, HSG has a lower sensitivity and specificity and high rates of false-positive and false-negative results compared to hysteroscopy. HSG is a poor test for uterine cavity evaluation.
- Hysterosonography (sonohysterography) is a combination of US with saline or contrast media infusion into the uterine cavity. Extension of this procedure to assess the patency of the fallopian tubes following examination of the uterine cavity is called hysterosalpingo-contrast sonography (HyCoSy). Hysterosonography improves delineation of the uterine cavity and is more accurate than US and HSG. It has high sensitivity (78%–100%) and specificity (71%–91%) for detecting intrauterine lesions [25, 27, 28]. As with US, it is more precise for diagnosing polyps or submucosal fibroids than endometrial hyperplasia or structural abnormalities [25, 27, 28]. Three-dimensional hysterosonography could also be performed and seems to be comparable to hysteroscopy for diagnosing intrauterine lesions.
- Hysteroscopy remains the most accurate test for intrauterine pathology [25, 27, 28] and is considered the gold standard [11]. Since hysteroscopy is an invasive method, it is usually reserved for further evaluation and treatment of

already-suspected anomalies [5, 11]. Hysteroscopy using a small-diameter hysteroscope allows this procedure to be conducted in the office setting, and simple polypectomy or adhesiolysis can be performed in the same setting. It allows visualization of the uterine cavity but not the uterine contour. Accordingly, diagnosing congenital uterine anomalies using hysteroscopy alone is insufficient. It should be investigated by MRI or 3D US.

Without suspected uterine pathology, a routine hysteroscopy before the first IVF cycle remains controversial. Though it may assist in identifying intrauterine pathology, it is questionable whether such information would affect treatment outcomes. Results of randomized trials have been mixed. Several studies have shown an improvement in pregnancy or live birth rates in patients undergoing hysteroscopy before their first IVF treatment. Others reported no benefits [29–31].

Fallopian tube pathology

There are several techniques to evaluate tubal integrity [11].

- *Hysterosalpingography:* Hysterosalpingography (HSG) is radiographic evaluation of the fallopian tubes that is performed by injecting radiocontrast of either oil-based or water-soluble media into the uterine cavity via the cervix. Contraindications to HSG include contrast allergy, pregnancy, and active pelvic infection. It should be performed during the early follicular phase of the menstrual cycle to ensure absence of pregnancy and to facilitate maximum uterine visibility. Post-HSG infection can occur in 0.3%–3.1% of cases, particularly in the presence of abnormal tubes [32].

HSG findings of “proximal tubal occlusion” are usually due to tubal spasm, collection of debris, or a mucus plug inside the proximal tubes. Such findings should be followed up with additional tests, such as selective tubal catheterization or laparoscopy. Hydrosalpinx is a result of a “distal tubal occlusion,” commonly caused by prior pelvic inflammatory disease, and it is characterized by dilatation of the tube without intraperitoneal spill [33]. It has been suggested that hydrosalpinx has a detrimental effect on the outcome of IVF [34]. According to a recent ASRM Practice Committee, tubal operation prior to IVF (either neosalpingostomy or salpingectomy) can improve pregnancy rates [35].

HSG sensitivity and specificity rates are 65% and 83%, respectively [36]. HSG is more specific for detecting distal as opposed to proximal occlusion [36] and has a high correlation (94%) with laparoscopic findings.

- *Hysterosalpingo-contrast sonography:* Hysterosalpingo-contrast sonography (HyCoSy) shows intratubal flow of contrast media. The presence of fluid in the cul-de-sac after uterine instillation implies patency of at least one tube. Pain induced by HyCoSy and its complications are comparable to HSG. Although HyCoSy might have been considered inferior to HSG for evaluating tubal patency, it has been shown to be as reliable as HSG in low-risk patients [37].
- *Laparoscopy:* Laparoscopy with chromoperturbation has long been considered the “gold standard” for evaluating tubal patency. Its advantages include the feasibility

to diagnose and treat conditions that decrease fertility, including endometriosis or periadnexal adhesions. However, it is an invasive procedure that requires general anaesthesia. The risk of major complications is low (<1%) [38]. Laparoscopy is indicated when there is evidence or strong suspicion of endometriosis, pelvic/adnexal adhesions, or significant tubal disease requiring treatment. In the era of ART, laparoscopy is rarely performed in the workup of infertility.

HSG and HyCoSy are the first-line tests to evaluate the fallopian tubes in infertile women [5]. These procedures are generally well tolerated, inexpensive, and capable of demonstrating tubal patency at rates as high as 80% [37]. The choice between these two techniques depends on availability, operator experience, and whether the patient is allergic to contrast media or iodine. Laparoscopy for diagnostic purposes is rarely needed.

Male investigation

Basic male infertility investigation begins with a detailed history and physical examination. Semen analysis and a serum hormonal profile represent the first-line laboratory investigations. The goal of these investigations is to identify the underlying causes of male factor infertility that can be corrected to enhance fertility. More importantly, a thorough male fertility evaluation may reveal serious associated conditions including testis cancer, osteoporosis/osteopenia, and genetic and hormone disorders that can have significant health consequences or even be life threatening.

History

A general history should include the developmental history, covering such things as congenital malformation of the genitalia, cryptorchidism, and delayed onset of puberty. Previous history of herniorrhaphy, particularly in childhood, may result in inadvertent damage to the vas deferens that has not been recognized. A history of mumps orchitis (particularly in adolescence), sexually transmitted infections, genitourinary surgeries, instrumentation, or trauma should be obtained. Symptoms of the lower urinary tract and erectile and ejaculatory functions should also be carefully reviewed.

A systematic review of related organ system function, such as pulmonary disease and upper respiratory infections, may suggest genetic conditions such as Young's syndrome, Kartagener's syndrome (immotile cilia syndrome; primary ciliary dyskinesia), or cystic fibrosis (CF). A history of a metabolic or neurological condition may be related to impaired erectile and ejaculatory function. History of gonadotoxic treatment should also be recorded. Use of medications, alcohol, drugs, and occupational and environmental exposure to toxins such as heat and chemicals that can act as endocrine disruptors should be recorded. Air pollution, for example, was found to be associated with lower sperm concentration and motility [39]. Heavy alcohol consumption can negatively affect spermatogenesis and sperm quality [40] and substance abuse can lead to impairment of the hypothalamic–pituitary–testicular axis [41]. Tobacco smoking, apart from decreasing sperm concentration and motility, can also have genetic and epigenetic effects [42].

Physical examination

A thorough physical examination should focus on general signs, such as secondary sex characteristics that reflect normal androgenization (hair distribution, absence of gynecomastia, and skeletal muscle development), and on the genitalia.

Genital examination includes localization of the penile urethral meatus and palpation of the testes for their presence, size, and consistency. Testicular cancer risk is increased significantly among men with infertility and is the most common type of cancer among young reproductive-aged males. Proper testicular examination may facilitate diagnosis. Testicular size can be assessed by using testis-shaped models of defined sizes (Prader orchidometer) and may be indicative of spermatogenesis. The normal range is 12–30 mL [43]. Small testes are related to testicular dysfunction or hypogonadism.

Size, texture, position, and orientation of the epididymis and the bilateral presence of the vasa should be carefully examined. Congenital bilateral absence of vas deferens (CBAVD) suggests the presence of mutation of the CF transmembrane conductance regular gene (CFTR). Cysts or nodularity of the epididymis suggest congenital or inflammatory changes that can lead to obstruction.

Examination of the spermatic cord in the upright position is important to evaluate the presence of varicocele. Varicocele is classified into three grades: (i) palpable only with Valsalva manoeuvre; (ii) palpable even without Valsalva manoeuvre; and (iii) detectable by visual inspection. Digital rectal examination can detect cysts in the seminal vesicles and prostatic adenoma and neoplasia.

Laboratory investigations

Semen analysis

Semen analysis (SA) is an essential component in the initial clinical evaluation of the male partner. The semen parameters may vary substantially from ejaculate to ejaculate; therefore, it is important to obtain at least two SAs at four weeks apart, especially if the first SA was abnormal [44]. The results from SA should be used to guide management of the patient. However, abnormal semen parameters could not by themselves predict if the patient will be fertile or not fertile [44]. As the number of abnormal parameters increases, the likelihood for infertility increases [44].

Semen samples should be ideally collected by masturbation after two to five days of abstinence [45]. However, recent studies show that shorter abstinence time (i.e. several hours) can improve sperm progressiveness and DNA fragmentation rate [46]. In exceptional circumstances, semen may be produced at home or during sexual intercourse using a special condom. How the sample was produced, difficulties in semen production, and any partial loss of the sample should be reported.

Semen analysis assesses parameters including volume, pH, sperm concentration, vitality, motility, and morphology. The main reference values of semen analysis according to the WHO are summarized in Table 37.1.

Interpreting semen analysis

Aspermia is the absence of semen and can be related to retrograde ejaculation or anejaculation due to psychological or neurological causes. In the case of retrograde ejaculation, a post-orgasm urine analysis should be performed, with specific preparation (alkalinization of urine) to evaluate sperm quality.

TABLE 37.1 Reference Values of Semen Analysis According to the World Health Organization (2010)

Criteria	Reference Value
Volume	≥1.5 mL
pH	≥7.2
Total sperm number	≥39 million/ejaculate
Sperm concentration	≥15 million/mL
Total motility	≥40%
Progressive motility	≥32%
Normal morphology	≥4%
Vitality	≥58%

Low semen volume may be associated with the absence or blockade of the seminal vesicles or the ejaculatory duct in the prostate. In men with congenital bilateral absence of the vas deferens (CBAVD), low semen volume is often seen due to the poor development of the seminal vesicles. Low semen volume can also be the result of a collection problem, androgen deficiency, obstruction to the ejaculatory duct, or partial retrograde ejaculation. High semen volume may reflect exudation in cases of active inflammation of the accessory organs.

The *pH* of the semen reflects the balance of pH from various accessory gland secretions, with the seminal vesicle secretions being alkaline, and prostatic secretions being acidic. A pH of less than 7 in a sample with low volume and azoospermia strongly suggests ejaculatory duct obstruction or CBAVD.

Oligospermia and azoospermia. Oligospermia is defined by a sperm density less than 15–20 million/mL and is considered severe when the sperm concentration is below 5 million/mL. Serum FSH and testosterone are recommended for men with oligospermia, and further endocrine and genetic evaluation (karyotype and Y-chromosome microdeletion) is indicated for men with severe oligospermia or azoospermia [46].

Azoospermia is defined by the absence of spermatozoa identified in the sample, and *cryptozoospermia* by the identification of spermatozoa only in the sediment of the semen post-centrifugation. Azoospermia is classified based on its aetiology as obstructive azoospermia (OA) or non-obstructive azoospermia (NOA).

Asthenospermia is used to describe reduced sperm motility. Sperm motility is graded as progressive motility (PR; spermatozoa moving actively regardless of the speed), non-progressive motility (NP; motility with an absence of progression), and immotile [47]. Reduced sperm motility suggests testicular or epididymal dysfunction and is associated with sperm autoantibodies, varicoceles, partial obstruction of the ejaculatory ducts, genital tract infections, and prolonged abstinence intervals. If the sperm is found to be viable but non-motile, the possibility of primary ciliary dyskinesia (Kartagener syndrome) should be considered [7].

Teratospermia is used to describe abnormal morphology of sperm cells. Morphological anomalies in spermatozoa could be identified in the head, neck, mid-piece, and tail. Morphological anomalies are commonly found in more than one part of a spermatozoon. Defective spermatogenesis and some epididymal pathologies may contribute to an increased percentage of

abnormal morphology of spermatozoa. Spermatozoa with abnormal morphology generally have a lower fertilizing potential, depending on the types of anomalies, and may also have abnormal DNA. Unfortunately, assessment of sperm morphology is associated with a number of technical difficulties related to variations in interpretation or poor performance in external quality control assessments.

Sperm vitality is an important variable, especially for samples with less than 40% progressively motile spermatozoa. The percentage of dead spermatozoa cannot exceed the percentage of immotile spermatozoa. Sperm viability is assessed using a dye test or a hypo-osmotic swelling (HOS) test [47]. Viable non-motile sperm identified using an HOS test can be used for intracytoplasmic sperm injection (ICSI).

Identification of non-sperm cells, such as epithelial cells or rounds cells (germ cells or leukocytes), may be indicative of a pathology of the efferent ducts (ciliary tufts), testicular damage (immature germ cells), or inflammation of the accessory glands (leukocytes). If the estimate of the round cell concentration exceeds 10^6 per mL, their nature should be assessed [47]. Special staining assessing their peroxidase activities could indicate that the round cells are leukocytes. Excessive numbers of leukocytes in the ejaculate may be associated with inflammation or infection. Leukocytes can impair sperm motility and DNA integrity through oxidative stress.

Sperm DNA fragmentation

Sperm chromatin quality or integrity ("DNA fragmentation") can be modified during spermatogenesis and transport through the reproductive tract. Although high levels of DNA fragmentation often correlate with poor semen parameters and infertile men, it is also found in men with normal semen parameters [48]. Damage in sperm DNA integrity can occur due to gonadotoxins, antidepressant use, heat exposure, radiation, genitourinary infection, and varicoceles. Several laboratory evaluations commonly used in basic science research have been gaining popularity in clinical research and practice for evaluating sperm DNA integrity. These include (i) sperm chromatin structure assay (SCSA), which tests abnormal chromatin structure as increased susceptibility of sperm DNA to acid-induced denaturation *in situ*; (ii) terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL) assay; and (iii) single-cell gel electrophoresis assay (Comet). Some investigators have suggested threshold values used to define an abnormal test for SCSA (25%–27%) and TUNEL assay (>36%) [45].

Low DNA fragmentation is significantly associated with increased likelihood of pregnancy *in vivo* and after intrauterine insemination [49]. Damage to sperm DNA integrity may contribute to poor reproductive performance in some couples and risk of spontaneous recurrent miscarriage. But this association is not strong enough to correctly predict outcomes of assisted reproduction, including IVF or ICSI [50], and to provide a clinical indication for the routine use of this test [44, 45]. According to the recent ASRM and American Urological Association, sperm DNA fragmentation analysis is not recommended as part of the initial evaluation of the infertile couple [44].

Ultrasound

Transscrotal US can be done to evaluate scrotal and inguinal pathologies (e.g. varicoceles and testicular mass). Transrectal US

TABLE 37.2 Male Hormonal Assessment Expected in Different Circumstances

	Testicular Failure	Obstructive Azoospermia	Hypogonadotropic Hypogonadism	Prolactin-Secreting Pituitary Tumour
FSH	↑	Normal	↓	↓ or Normal
LH	↑ or Normal	Normal	↓	↓ or Normal
Testosterone	↓ or Normal	Normal	↓	↓
Prolactin	Normal	Normal	Normal	↑

can be done to assess the prostate, ejaculatory ducts, and seminal vesicles (for cystic lesions or obstruction). Renal US is indicated for men with unilateral or bilateral vasal agenesis. US is not done routinely in male fertility evaluation. Its goal is to confirm a pathology that was suspected during physical examination or was suggested based on semen and hormonal analysis.

Endocrine tests

If the semen analysis indicates a low concentration of sperm, or there is male sexual dysfunction, further endocrine tests should be requested. Serum FSH and total testosterone measurements should be performed in all cases of oligospermia. This will help distinguish between pituitary–hypothalamic axis dysfunction, testicular dysfunction, and reproductive tract obstruction. LH and prolactin are indicated for men with low serum testosterone (<300 ng/dL). Prolactin is also indicated in men with hypogonadotropic hypogonadism or decreased libido.

Low levels of FSH, LH, and testosterone in the context of low sperm concentrations suggest hypogonadotropic hypogonadism. Though it is not a common cause of male infertility, this endocrinopathy may be a result of Kallmann's syndrome or acquired causes as hyperprolactinemia and hemochromatosis. If testicular failure mainly impairs the spermatogenesis and not endocrine function, testosterone, FSH, and LH levels may be within normal limits. In the case of complete testicular failure, FSH and LH will be elevated, whereas testosterone will be normal or low (Table 37.2).

Genetic testing

Non-obstructive azoospermia (NOA) and severe oligospermia

Males having abnormal spermatogenesis related to testicular failure, such as in NOA or severe oligospermia, are at increased risk of having genetic abnormalities compared to fertile men [45]. Genetic testing, including karyotype analysis and Y-chromosome microdeletion, is recommended in these circumstances before performing ICSI [45]. A karyotype analysis can diagnose chromosomal abnormalities (e.g. Klinefelter's syndrome [KS]) or other chromosomal structure abnormalities (e.g. Robertsonian or reciprocal translocations). KS is the most common chromosomal abnormality: non-mosaic KS accounts for 11% of azoospermia cases and mosaic KS accounts for 0.5% of severe oligospermia cases [50]. If the karyotype is abnormal, there is an increased risk of sperm chromosomal aneuploidy, and genetic counselling including pre-implantation genetic diagnosis should be discussed with the couples prior to assisted reproduction. Y-chromosome

microdeletion can severely impair spermatogenesis and can be transmitted to the offspring. Thus, genetic counselling is important for these men.

Obstructive azoospermia

Men with congenital obstructive azoospermia should be tested for cystic fibrosis (CF), since there is a strong association between CBAVD and mutations of the CFTR gene; almost all men with CF exhibit CBAVD. Of men with CBAVD, more than 50% are heterozygous for the CFTR gene mutation or carry compound heterozygous mutations including milder coding mutations for the CFTR gene [52]. The CFTR mutation is also linked to congenital unilateral agenesis of the vas deferens (CUAVD) and with congenital epididymal obstruction [53]. The cumulative carrier frequency varies according to ethnicity. A carrier frequency as high as 1 in 25 is seen in men who are Northern European descendants or Ashkenazi Jewish.

In case of agenesis of the vas deferens related to CFTR mutation, a history of non-severe pulmonary diseases or asthma may or may not be present. Concerning the genitalia, fibrous cord-like vas may be palpable, only the seminal vesicles and proximal vas may be missing, or asymmetry may be apparent [49].

CFTR screening of the female partner is essential. There is a 25% risk of an affected offspring if both male and female partners are carriers of a mutation in the CFTR gene, and up to 50% risk if the female is a carrier and the male has mutations in both alleles [44]. When CFTR mutations are found in both partners, pre-implantation genetic testing may be proposed to the couple to prevent the birth of a child with CF.

In summary, men with NOA or severe oligospermia should be offered karyotype evaluation and Y-chromosome analysis. CBAVD/CUAVD further warrants CFTR mutation screening and genetic counselling.

Conclusion

Assessment and management of the infertile couple is a stepwise process of initial evaluation, further investigation when necessary, and consultation regarding treatment options (Figure 37.1). It is highly recommended that both partners will undergo evaluation in parallel to optimize treatment success [44]. It is known that failure to achieve pregnancy increases the psychological stress for the couples [54]. Our goal as physicians is to provide education, counselling, and assistance, including emotional support, during the initial investigations and later during the treatment.

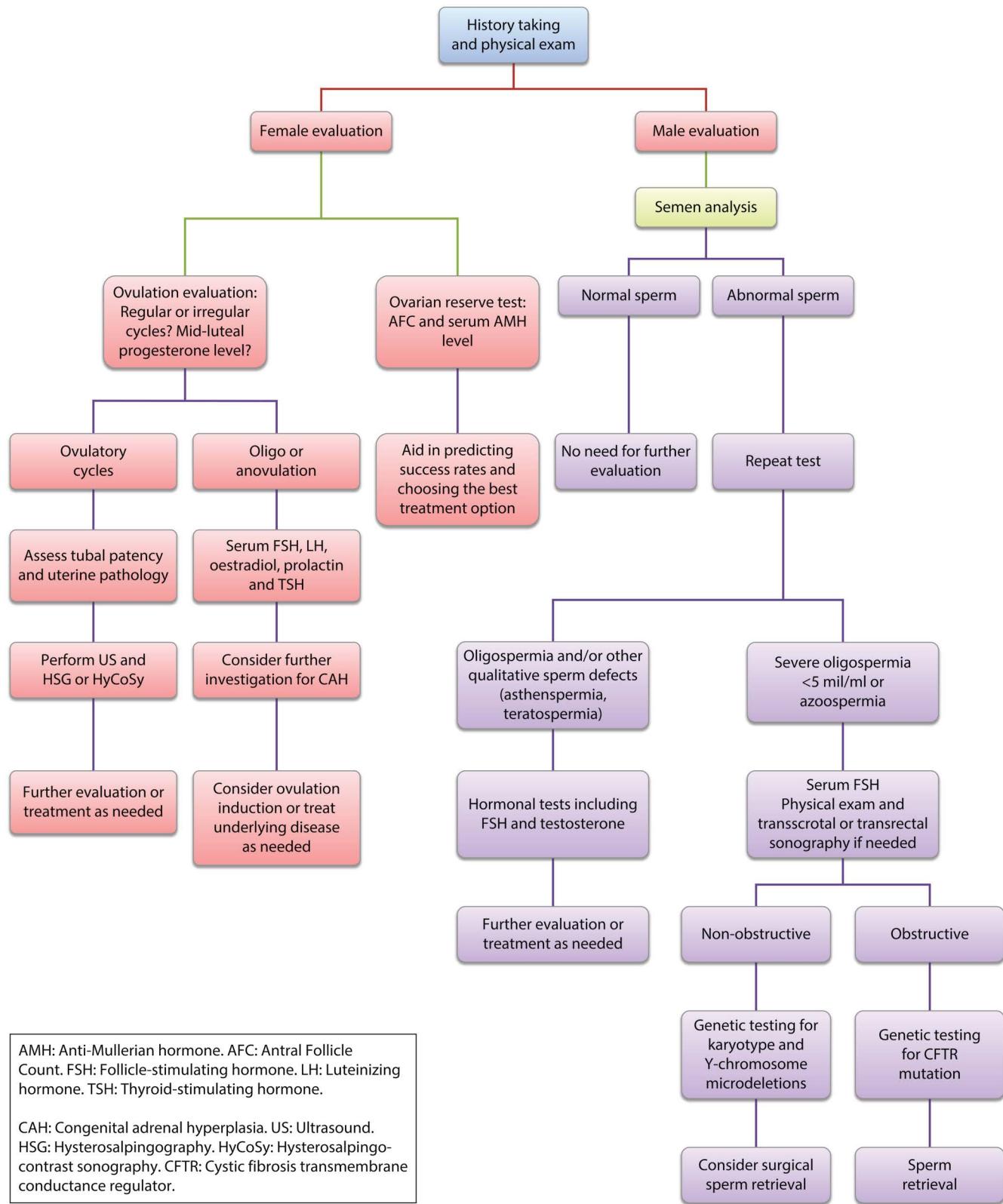


FIGURE 37.1 Evaluation of the infertile couple.

References

1. Practice Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss. *Fertil Steril.* 2008;90(Suppl 5):S60.
2. European Society of Human Reproduction and Embryology. ESHRE: A policy audit on fertility: Analysis of 9 EU countries; March 2017. Available at: <https://www.esore.eu/Press-Room/Resources>.
3. Practice Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss: A committee opinion. *Fertil Steril.* 2020;113(3):533–5. doi: [10.1016/j.fertnstert.2019.11.025](https://doi.org/10.1016/j.fertnstert.2019.11.025).
4. Thonneau P, Marchand S, Tallec A, et al. Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988–1989). *Hum Reprod.* 1991; 6(6): 811–6.
5. NICE: National Institute for Health and Clinical Excellence. Fertility assessment and treatment for people with fertility problems. NICE Clinical Guideline. 2013;156.
6. Hull MG, Glazener CM, Kelly NJ, et al. Population study of causes, treatment, and outcome of infertility. *Br Med J.* 1985;291(6510):1693–7.
7. Male infertility. In: Speroff's Clinical Gynecologic Endocrinology and Infertility, 9th ed. Hugh ST, Lubna P, Emre S, (eds.). New Haven, Connecticut: Lippincott Williams & Wilkins (LWW), chapter 26, pp. 1028–66, 2019.
8. CFAS Consensus Document for the Investigation of Infertility. 2002 Aug. Available at: https://cfas.ca/_Library/clinical_practice_guidelines/CFAS-Consensus-Policy.pdf
9. Douglas Wilson R, Van Mieghem T, Langlois S, Church P. Guideline no. 410: Prevention, screening, diagnosis, and pregnancy management for fetal neural tube defects. *J Obstet Gynaecol Can.* 2021;43(1):124–39.
10. Maxwell C, Gaudet L, Cassir G, Nowik C, McLeod NL, Jacob C, Walker M. Guideline no. 391-pregnancy and maternal obesity part 1: Pre-conception and prenatal care. *J Obstet Gynaecol Can.* 2019;41(11):1623–40.
11. Practice Committee of the American Society for Reproductive Medicine. Diagnostic evaluation of the infertile female: A committee opinion. *Fertil Steril.* 2015;103(6):e44–50.
12. Munster K, Schmidt L, Helm P. Length and variation in the menstrual cycle—A cross-sectional study from a Danish county. *Br J Obstet Gynaecol.* 1992;99(5):422–9.
13. Guermandi E, Vegetti W, Bianchi MM, Ugliesti A, Ragni G, Crosignani P. Reliability of ovulation tests in infertile women. *Obstet Gynecol.* 2001;97(1):92–6.
14. McGovern PG, Myers ER, Silva S, et al. Absence of secretory endometrium after false-positive home urine luteinizing hormone testing. *Fertil Steril.* 2004;82(5):1273–7.
15. Wathen NC, Perry L, Lilford RJ, Chard T. Interpretation of single progesterone measurement in diagnosis of anovulation and defective luteal phase: Observations on analysis of the normal range. *Br Med J.* 1984;288(6410):7–9.
16. Dewailly D, Andersen CY, Balen A et al. The physiology and clinical utility of anti-mullerian hormone in women. *Hum Reprod Update.* 2014;20(3):370–85.
17. Broer SL, van Disseldorp J, Broeze KA et al. Added value of ovarian reserve testing on patient characteristics in the prediction of ovarian response and ongoing pregnancy: An individual patient data approach. *Hum Reprod Update.* 2013;19(1):26–36.
18. Broekmans FJ, Kwee J, Hendriks DJ, Mol BW, Lambalk CB. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update.* 2006;12(6):685–718.
19. Practice Committee of the American Society for Reproductive Medicine. Testing and interpreting measures of ovarian reserve: A committee opinion. *Fertil Steril.* 2020;114(6):1151–7.
20. Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L. ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: The Bologna criteria. *Hum Reprod.* 2011;26(7):1616–24.
21. Akhtar MA, Saravelos SH, Li TC, Jayaprakasan K; Royal College of Obstetricians and Gynaecologists. Reproductive implications and management of congenital uterine anomalies: Scientific Impact Paper No. 62 November 2019. *BJOG.* 2020 Apr;127(5):e1–13.
22. Katz Z, Ben-Arie A, Lurie S, Manor M, Insler V. Beneficial effect of hysteroscopic metroplasty on the reproductive outcome in a 'T-shaped' uterus. *Gynecol Obstet Invest.* 1996;41(1):41–3.
23. Di Spiezo Sardo A, Florio P, Nazzaro G, Spinelli M, Paladini D, Di Carlo C, et al. Hysteroscopic outpatient metroplasty to expand dysmorphic uteri (HOME-DU technique): A pilot study. *Reprod Biomed Online.* 2015;30(2):166–74.
24. Bonnamy L, Marret H, Perrotin F, Body G, Berger C, Lansac J. Sonohysterography: A prospective survey of results and complications in 81 patients. *Eur J Obstet Gynecol Reprod Biol.* 2002; 102(1):42–7.
25. Grimbizis GF, Tsolakidis D, Mikos T, et al. A prospective comparison of transvaginal ultrasound, saline infusion sonohysterography, and diagnostic hysteroscopy in the evaluation of endometrial pathology. *Fertil Steril.* 2010;94(7):2720–5.
26. Pleş L, Alexandrescu C, Ionescu CA, Arvătescu CA, Vladareanu S, Moga MA. Three-dimensional scan of the uterine cavity of infertile women before assisted reproductive technology use. *Medicine (Baltimore).* 2018;97(41):e12764.
27. Bingol B, Gunenc Z, Gedikbasi A, Guner H, Tasdemir S, Tiraz B. Comparison of diagnostic accuracy of saline infusion sonohysterography, transvaginal sonography and hysteroscopy. *J Obstet Gynaecol.* 2011;31(1):54–8.
28. Soares SR, Barbosa dos Reis MM, Camargos AF. Diagnostic accuracy of sonohysterography, transvaginal sonography, and hysterosalpingography in patients with uterine cavity diseases. *Fertil Steril.* 2000;73(2):406–11.
29. Pundir J, Pundir V, Omanwa K, Khalaf Y, El-Toukhy T. Hysteroscopy prior to the first IVF cycle: A systematic review and meta-analysis. *Reprod Biomed Online.* 2014;28(2):151–61.
30. Elsetohy KA, Askalany AH, Hassan M, Dawood Z. Routine office hysteroscopy prior to ICSI vs. ICSI alone in patients with normal transvaginal ultrasound: A randomized controlled trial. *Arch Gynecol Obstet.* 2015;291(1):193–9.
31. Smit JG, Kasius JC, Ejikemans MJ, Koks CA, Van Golde R, Nap AW, et al. Hysteroscopy before in vitro fertilization (in SIGHT): A multicenter randomized controlled trial. *Lancet.* 2016;387(10038):2622–29.
32. Stumpf PG, March CM. Febrile morbidity following hysterosalpingography: Identification of risk factors and recommendations for prophylaxis. *Fertil Steril.* 1980;33(5):487–92.
33. Grigovich M, Kacharia VS, Bharwani N, Hemingway A, Mijatovic V, Rodgers SK. Evaluating fallopian tube patency: What the radiologist needs to know. *Radiographics.* 2021 Oct;41(6): 1876–96.
34. Johnson NP, Mak W, Sowter MC. Surgical treatment for tubal disease in women due to undergo in vitro fertilisation. *Cochrane Database Syst Rev* 2004;(3):CD002125.
35. Practice Committee of the American Society for Reproductive Medicine. Role of tubal surgery in the era of assisted reproductive technology: A committee opinion. *Fertil Steril.* 2021 May;115(5):1143–50.
36. Swart P, Mol BW, van der Veen F, van Beurden M, Redekop WK, Bossuyt PM. The accuracy of hysterosalpingography in the diagnosis of tubal pathology: A meta-analysis. *Fertil Steril.* 1995;64(3):486–91.
37. Saunders RD, Shwayder JM, Nakajima ST. Current methods of tubal patency assessment. *Fertil Steril.* 2011;95(7):2171–9.
38. Chapron C, Querleu D, Bruhat MA et al. Surgical complications of diagnostic and operative gynaecological laparoscopy: A series of 29,966 cases. *Hum Reprod.* 1998;13(4):867–72.
39. Qiu Y, Yang T, Seyler BC, et al. Ambient air pollution and male fecundity: A retrospective analysis of longitudinal data from a Chinese human sperm bank (2013–2018). *Environ Res.* 2020;186:109528.

40. Ramírez N, Estofán G, Tissera A, Molina R, Luque EM, Torres PJ, Mangeaud A, Martini AC. Do aging, drinking, and having unhealthy weight have a synergistic impact on semen quality? *J Assist Reprod Genet.* 2021;38(11):2985–94.
41. Ajayi AF, Akhigbe RE. The physiology of male reproduction: Impact of drugs and their abuse on male fertility. *Andrologia.* 2020 Oct;52(9):e13672.
42. Omolaoye TS, El Shahawy O, Skosana BT, Boillat T, Loney T, du Plessis SS. The mutagenic effect of tobacco smoke on male fertility. *Environ Sci Pollut Res Int.* 2021 Sep 18;29(41):62055–66.
43. Lanfranco F, Kamischke A, Zitzmann M, Nieschlag E. Klinefelter's syndrome. *Lancet.* 2004;364(9430):273–83.
44. Schlegel PN, Sigman M, Collura B, et al. Diagnosis and treatment of infertility in men: AUA/ASRM guideline part I. *Fertil Steril.* 2021;115(1):54–61.
45. Practice Committee of the American Society for Reproductive Medicine. Diagnostic evaluation of the infertile male: A committee opinion. *Fertil Steril.* 2015;103(3):e18–25.
46. Dahan MH, Mills G, Khoudja R, Gagnon A, Tan G, Tan SL. Three hour abstinence as a treatment for high sperm DNA fragmentation: A prospective cohort study. *J Assist Reprod Genet.* 2021;38(1):227–33.
47. WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th ed. Cambridge, UK: Cambridge University Press, 2010.
48. Schulte RT, Ohl DA, Sigman M, Smith GD. Sperm DNA damage in male infertility: Etiologies, assays, and outcomes. *J Assist Reprod Genet.* 2010;27(1):3–12.
49. Evenson D, Wixon R. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. *Reprod Biomed Online.* 2006;12(4):466–72.
50. Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? *Fertil Steril.* 2008;89(4):823–31.
51. Van Assche E, Bonduelle M, Tournaye H et al. Cytogenetics of infertile men. *Hum Reprod.* 1996; 11(Suppl 4): 1–24; discussion 5–6.
52. Dork T, Dworniczak B, Aulehla-Scholz C et al. Distinct spectrum of CFTR gene mutations in congenital absence of vas deferens. *Hum Genet.* 1997;100(3–4):365–77.
53. Lissens W, Mercier B, Tournaye H et al. Cystic fibrosis and infertility caused by congenital bilateral absence of the vas deferens and related clinical entities. *Hum Reprod.* 1996;11(Suppl 4):55–78; discussion 79–80.
54. Yoldemir T, Yassa M, Atasayan K. Comparison of anxiety scores between unexplained primary and secondary infertile couples. *Gynecol Endocrinol.* 2021 Nov;37(11):1008–13.

38

PROGNOSTIC TESTING FOR OVARIAN RESERVE

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Female reproductive aging

Age-related sub-fertility and ovarian reserve

With the postponement of childbearing in Western societies, rates of sub-fertility related to advanced female age have increased considerably [1]. A higher proportion of couples therefore depends on assisted reproduction technology (ART) to achieve a pregnancy. The increase of sub-fertility with advanced female age is mainly based on changes in ovarian function referred to as decreased or diminished ovarian reserve. Ovarian reserve can be defined as the quantity and the quality of the remaining oocytes in both ovaries at a given age. Declines in follicle numbers dictate the occurrence of irregular cycles and ultimately the cessation of menstrual bleeding (i.e. menopause), whereas oocyte quality decay results in decreasing fertility, defined as the capacity to conceive and give birth to a child (Figure 38.1) [2].

Variability of reproductive aging

There is substantial individual variation in the onset of menopause, varying roughly between 40 and 60 years, with a mean age of 51 years. This variation has shown to be rather constant over time and populations worldwide [3–5]. Female fecundity is believed to decrease after the age of 31 years, a decrease that may accelerate after 37 years of age, leading to sterility at a mean age of 41 years of age [6]. As is the case with menopause, the rate of decline in fertility may vary considerably between women of the same age, e.g. a woman at the age of 35 years either may be close to natural sterility or have a normal fertility comparable to a 25-year-old. The decrease of female fertility is believed to exhibit the same range of variation as for the occurrence of menopause [7]. This implies that age at menopause is considered a proxy variable for age at loss of natural fertility, with a fixed time period of 10 years in between. The correct prediction of menopause in an individual woman would therefore provide valuable information regarding a woman's fertile lifespan and hence aid in preventing future sub-fertility (Figure 38.2).

Still, the presumed relationship between quantity of follicles and quality at the oocyte level may be more complicated. The variation in fecundity within female age groups is notable. Moreover, within quantity groups, defined according to markers such as the antral follicle count (AFC) or anti-Müllerian hormone (AMH) level, fertility is highly influenced by the age of the female. Unfortunately, studies that address the variation of female fertility depending on both age and quantitative ovarian reserve status are lacking, due to the fact that simple tests of qualitative ovarian reserve (i.e. embryo quality) are not present at the current time [8].

Age-related decline in natural and assisted fertility

The human species can be considered as relatively sub-fertile compared to other animals [10, 11]. The average monthly fecundity rate of approximately 20% implies that among human couples trying to conceive, many exposure months may be needed to achieve their goal, especially if monthly fecundity has dropped with increasing female age [12].

The proportion of infertile couples (by definition the failure to achieve a vital pregnancy within one year) will mount to 10%–20% in the age group of women over 35 years, compared to only 4% for women in their twenties. These infertility rates may rise to 30%–50% for only moderately fertile women of age 35 years and over, which may lead to trying to conceive for several years without any result [12, 13]. Maintaining regular menstrual cycles when age-related natural fecundity has already been reduced to approximately zero means that women are largely unaware that this process is taking place.

The age-related decline in female fertility has also been shown in numerous reports concerning *in vitro* fertilization (IVF) programs. After a mean female age of approximately 34 years, the chance of producing a live birth in IVF programs decreases steadily and reduces to less than 10% per cycle in women over 40 years of age (Figure 38.3). The chance of a live birth after IVF depends on both the quantitative and qualitative ovarian reserve. A reduced quantitative ovarian reserve is expressed by a poor response to ovarian stimulation. The qualitative aspect is best expressed by female age. A young woman with a poor response to controlled ovarian stimulation (COS) may have a reduced quantitative ovarian reserve, but as the quality aspect of her ovarian reserve is still good, she will still have reasonable pregnancy prospects. By contrast, an older woman with a poor response has a reduced quantitative and qualitative ovarian reserve and therefore her prospects of becoming pregnant after ART use are very poor [8, 14].

Ovarian reserve prediction

The knowledge and insights into the process of ovarian aging imply that for ovarian reserve testing prior to IVF, female age remains the predictor of first choice. The availability of a test to be capable of providing reliable information regarding a woman's individual ovarian reserve within a certain age category would enable the clinician to provide an individually tailored treatment plan. For instance, a reliable test would be helpful in counselling women with a low ovarian reserve regarding their chances of conceiving or alternatively of preserving oocytes. In the case of older infertile women seeking treatment, the test could allow older women with a still sufficient quantitative ovarian reserve to start IVF treatment, while for such cases with an exhausted reserve, refusal of IVF could be proposed. Ultimately, the observed response to maximal ovarian stimulation may provide the most accurate information on the reserve capacity of the ovaries. In the following two sections, the biological rationale behind ovarian reserve testing and the accuracy and clinical value (in tailoring treatment) of several of these tests are discussed.

The physiological background to ovarian reserve testing

In general, as outlined before, age of the woman is a simple way of obtaining information on the extent of her ovarian reserve, regarding both quantity as well as quality [15]. However, because

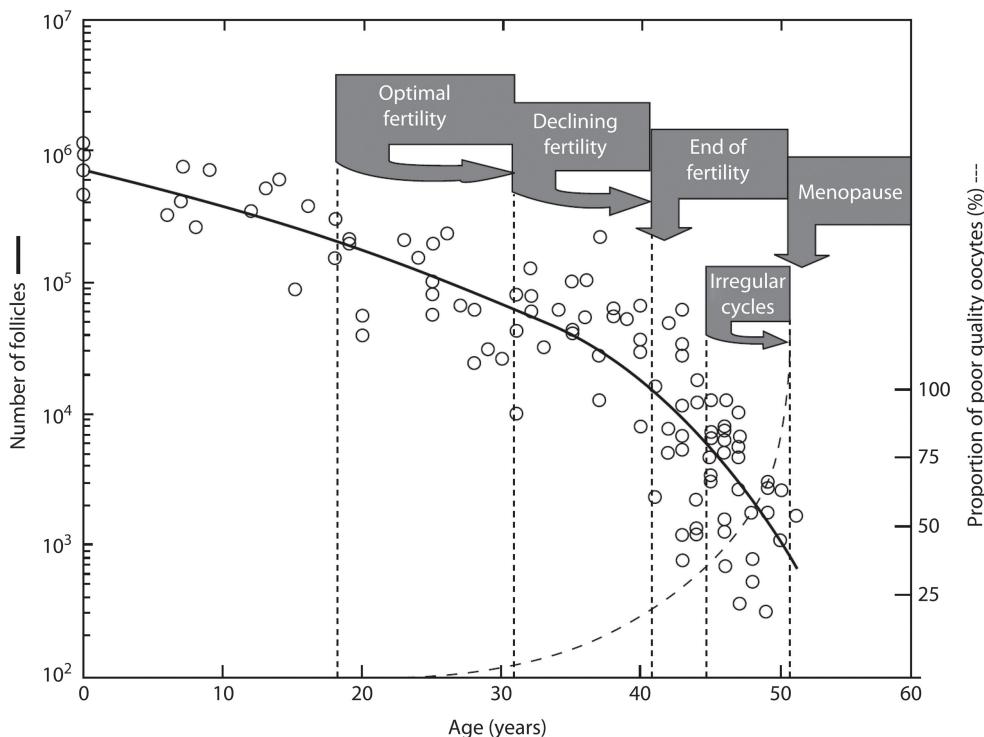


FIGURE 38.1 Quantitative (solid line) and qualitative (dotted line) declines of the ovarian follicle pool, which is assumed to dictate the onset of important reproductive events. (Adapted from [9].)

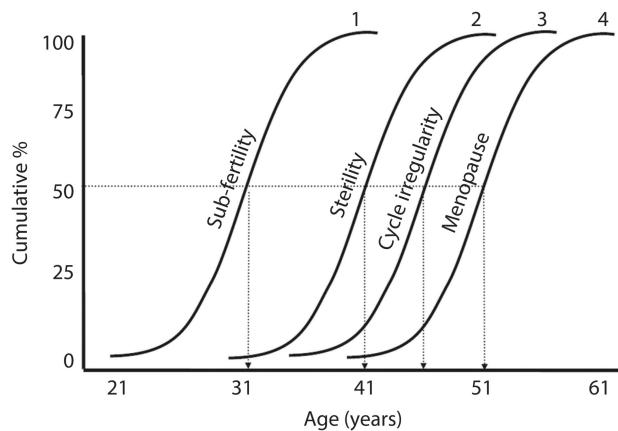


FIGURE 38.2 Variations in age at the occurrence of specific stages of ovarian aging. (For explanation of the background of data, see [2], with permission.)

of the substantial variation between women of the same age category, female age is not sufficient.

It would therefore be useful to identify young women with evident accelerated ovarian aging or older women with still adequate ovarian reserve. If it would be possible to identify such women, fertility management could be effectively individualized. For instance, stimulation dose or treatment scheme could be adjusted [16], counselling against initiation of IVF treatment or pertinent refusal could be effected, or treatment could be initiated early before the reserve has diminished too far.

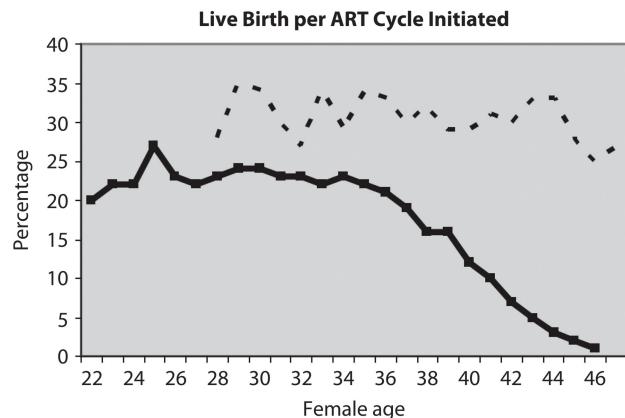


FIGURE 38.3 Effect upon average singleton live birth rates of female age, showing a steady decrease after the age of 34 years. The dotted line represents the average singleton live birth rate after oocyte donation as a function of the recipient age. It underlines the potential of oocyte donation in the treatment of women who remained unsuccessful in previous *in vitro* fertilization treatment. Abbreviation: ART, assisted reproduction technology. (Data were drawn from the 2003 CDC ART report [<http://www.cdc.gov/art/>].)

Ovarian reserve tests (ORTs) and their valuation

Most tests examined in the literature aim to predict specific outcomes related to ovarian reserve. The preferred or gold standard outcome of prediction studies would be live birth after a series of

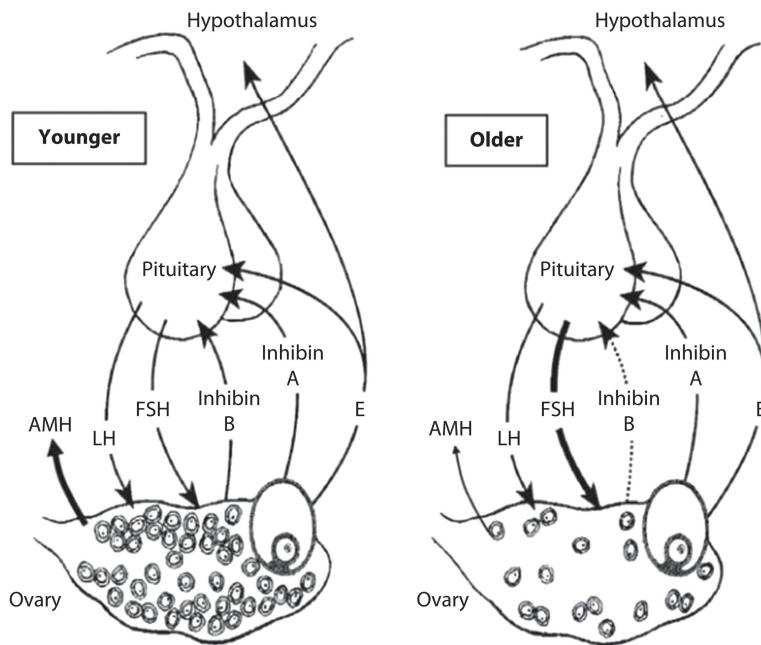


FIGURE 38.4 Illustration of the changes in follicle reserve with increasing female age and the effects of these quantitative changes upon several endocrine factors. Abbreviations: AMH, anti-Mullerian hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone. (Adapted from [21].)

ART exposure cycles, but other outcomes (especially oocyte yield or follicle number and pregnancy after one IVF/intracytoplasmic sperm injection [ICSI] cycle) are in fact the most common. As the occurrence of pregnancy in a single exposure to IVF and embryo transfer will be dependent on many other factors besides ovarian reserve, like laboratory performance and transfer technique, focus has been mostly upon the capacity of these tests to predict the ovarian response. Indeed, most if not all ovarian reserve tests (ORTs) relate to the size of the follicle cohort that is at any time responsive to FSH. The AFC assessed by transvaginal ultrasonography provides direct visual assessment of the cohort [17]. The endocrine marker AMH, which is produced by the granulosa cells surrounding the antral follicles, provides a direct marker of quantity [18, 19].

Baseline FSH, which has been extensively studied in the past decades, provides the most indirect marker. FSH levels increase with advancing age due to a reduction in the release of inhibin B and oestradiol, thereby reducing the negative feedback on FSH release from the pituitary (Figure 38.4) [20]. High FSH levels therefore represent small cohort sizes.

The clinical value of ovarian reserve testing

ART treatment outcome prediction

Poor-response prediction

In the scenario of IVF treatment, ovarian reserve can be considered normal in conditions where stimulation with the use of exogenous gonadotropins will result in the development of some 5–15 follicles and the retrieval of a corresponding number of healthy oocytes at follicle puncture [22, 23]. With such a yield, the chances of producing a live birth through IVF are considered optimal [24].

The Bologna criteria have been defined as a consensus regarding the criteria of a poor responder. The criteria state that after a poor response to COS, a woman can be classified as a poor

responder (i.e. with a diminished ovarian reserve) when at least two of the following three features are present: (i) advanced maternal age or any other risk factor for poor ovarian response (POR); (ii) a previous POR; and (iii) an abnormal ORT. Two episodes of POR after maximal stimulation are sufficient to define a patient as a poor responder in the absence of advanced maternal age or abnormal ORT [25].

A poor responder will generally be interpreted as a proof of diminished ovarian reserve and reduced prognosis for pregnancy. For that reason, poor-response prediction has been studied extensively, although mainly in relatively small studies. In 2011, an international project was undertaken to combine all of these smaller studies and merge them into one large summary database. With all of these data combined, a more robust analysis could be performed and more solid answers regarding the value of ovarian reserve testing could be given. Such a study set-up is called an individual patient data meta-analysis (IPD-MA), and this is regarded as the gold standard for test evaluations.

The IPD-MA for response and pregnancy outcome prediction after ART treatment included 5705 women undergoing their first IVF cycle. It appeared that the AFC and AMH are superior over the other ORTs, especially basal FSH, in the prediction of a poor response. AMH and the AFC are adequate predictors of a poor ovarian response to COS in IVF, with areas under the curve–receiver–operator characteristic curve (AUC–ROC) of 0.78 and 0.76, respectively (Figure 38.5) [26].

Multivariable analyses showed that a model with age, AFC, and AMH had a significantly higher predictive accuracy than a model based on age alone (AUC–ROC 0.80 vs. 0.61, $p \leq 0.001$), thereby confirming that AFC and AMH have added value to female age in the prediction of a poor response. Interestingly, AMH alone yielded an accuracy that is comparable to all multivariable models, suggesting that a single measurement of AMH would be sufficient [26].

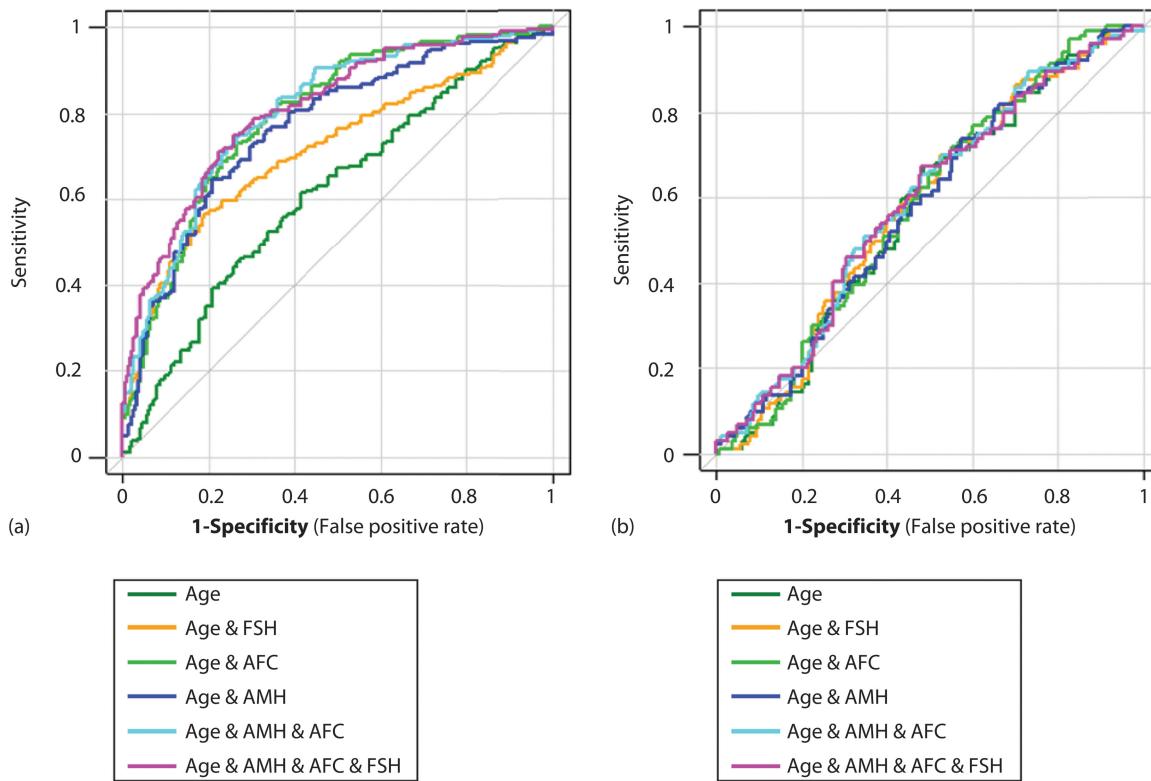


FIGURE 38.5 Receiver–operator characteristic (ROC) curves of age and ovarian reserve tests (ORTs) in the prediction of poor response and ongoing pregnancy. (a) Poor-response prediction based on age and ORT. The ROC curves of age or age combined with one or more ORT are depicted. The ROC curves for “Age + AMH,” “Age + AMH + AFC,” and “Age + AMH + AFC + FSH” run towards the upper-left corner, indicating a good capacity to discriminate between normal and poor responders at certain cut-off levels. (b) Ongoing pregnancy prediction based on age and ORT. The ROC curves for age or age combined with one or more ORT run almost parallel to or even cross the x/y line, indicating that the tests are useless for pregnancy prediction. Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone. (Adapted from [26].)

If poor response was to be the endpoint of interest, then the clinical value of these tests would be satisfactory. Unfortunately, though, no proven strategy to prevent the occurrence of poor response is currently known. Also, a poor response may not always imply a poor prognosis, especially in younger women [27]. The same may be true for “poor responders” after the application of mild stimulation protocols [28]. In poor responders to a first IVF cycle, it has become increasingly clear that any adaptation in the treatment protocol in a second cycle will improve neither the subsequent response nor the prognosis for pregnancy where randomized trials are concerned [29]. Tailoring treatment to aim for better ART outcomes will be discussed in the section “Applicability of ORTs.”

Excessive-response prediction

Excessive responders may be in jeopardy due to high patient discomfort, reduced pregnancy rates, and ovarian hyperstimulation syndrome risks [30, 31]. In view of these drawbacks, elimination of exaggerated ovarian response in stimulation protocols will improve safety, success, and cost factors of ART programs.

A separate IPD meta-analysis was performed to study excessive response prediction. This IPD-MA included 4786 women undergoing their first IVF cycle. This study showed that both AMH and the AFC are accurate predictors of excessive response to COS,

with AUC-ROC values for AMH and the AFC of 0.81 and 0.79, respectively [32].

Multivariable analyses showed an increase in the AUC-ROC from 0.61 to 0.85 when, besides age, the ORTs of the AFC and AMH were added, thereby confirming the added value of the AFC and AMH. For excessive-response prediction, the combination of the AFC with AMH is superior to a single ORT (Figure 38.6) [32]. The clinical value of excessive-response prediction and the possibilities to tailor treatment will be discussed in the section “Applicability of ORTs.”

Pregnancy prediction

The IPD-MA also studied the value of ORTs for the prediction of ongoing pregnancy after IVF. For these analyses, 5705 women undergoing their first IVF cycle could be included. The predictive ability for the occurrence of pregnancy after IVF was very small.

In the multivariable analysis, it became clear that ORTs do not have any added value in the prediction of ongoing pregnancy to female age alone (Figure 38.5). Age alone has a moderate AUC-ROC of 0.57. When combining age with AMH and AFC, the AUC-ROC is 0.59 [26]. Neither combination of ORTs could improve this accuracy. Therefore, ORTs are not useful in the prediction of ongoing pregnancy after IVF.

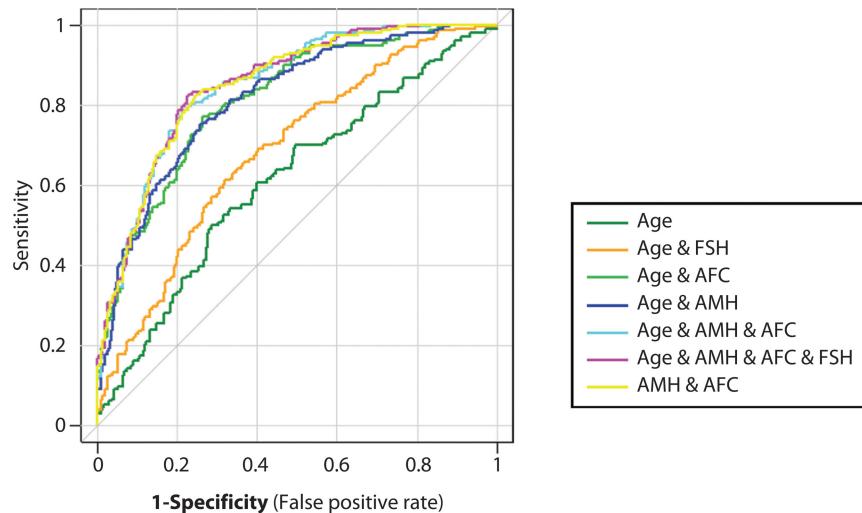


FIGURE 38.6 Receiver-operator characteristic (ROC) curves of age and ovarian reserve tests in the prediction of an excessive response. The ROC curves of age and age combined with one or more ovarian reserve test are depicted. The ROC curves for “Age + AMH,” “Age + AFC,” “Age + AMH + AFC,” and “Age + AMH + AFC + FSH” run towards the upper-left corner of the ROC space, indicating a good capacity to discriminate between normal and excessive responders at certain cut-off levels. Abbreviations: AFC, antral follicle count; AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone. (Adapted from [26].)

This finding should not be regarded as a surprise, as most tests relate to the quantitative aspects of the ovarian reserve that are constantly present (i.e. antral follicle cohort size), while the quality perspective is only tested against a single exposure, which certainly will not be a good expression of a couple’s fertility potential (this can only be tested properly in a series of ART cycles).

However, recent studies have noted that, although ovarian reserve markers may not predict pregnancy, they can be used to make a moderate distinction between patients with a good and poor prognosis. The success of IVF was found to mainly depend on maternal age and serum AMH concentrations [33]. Specifically, in older women, ORTs such as AMH or the AFC could help with identifying couples where refraining from treatment is the best advice, such as in favour of egg donation or adoption (Table 38.1) [8].

Applicability of ORTs; tailoring treatment in ART practice

Since it has been demonstrated that ORTs are adequate predictors of a poor and excessive response, the focus of interest has shifted to the possibility of tailoring treatment on an individual level to improve treatment outcomes, not only in pregnancy prospects but also in safety aspects.

Prior to the start of the first IVF cycle, ORTs could be used to determine the FSH dosage of the first IVF cycle. Thus far, several studies exist on the effect of adapting the dosage of FSH based on prior ORTs to obtain an optimal number of oocytes and improve prospects for pregnancy. A first study showed that predicted poor responders, based on an AFC of below 5, did not benefit from a higher starting dosage of gonadotropins in the first IVF treatment

TABLE 38.1 Predicted One-Year Probability of Achieving a Live Birth According to a Simplified Model Based on the Data of All Patients

Age (Years)	AMH ($\mu\text{g/L}$)					Total No. of Patients
	0–1	1–2	2–3	3–5	5–25	
0–30	0.44 (0.39–0.48)	0.54 (0.50–0.58)	0.56 (0.53–0.60)	0.68 (0.65–0.70)	0.68 (0.65–0.71)	67
n	3	14	15	16	19	
30–35	0.41 (0.35–0.45)	0.51 (0.46–0.55)	0.53 (0.49–0.57)	0.64 (0.61–0.67)	0.64 (0.61–0.67)	182
n	34	43	48	37	20	
35–40	0.32 (0.26–0.38)	0.41 (0.36–0.46)	0.43 (0.38–0.48)	0.53 (0.49–0.57)	0.54 (0.50–0.57)	192
n	61	61	30	22	18	
40–45	0.16 (0.08–0.23)	0.21 (0.14–0.27)	0.22 (0.15–0.28)	0.29 (0.22–0.34)	0.29 (0.22–0.34)	46
n	23	9	7	5	2	
Total no. of patients	121	127	100	80	59	487

Source: From [8], with permission.

Note: Probability values are presented with 95% confidence intervals. Abbreviation: AMH, anti-Mullerian hormone.

cycle [34]. Later, the OPTIMIST trial studied the effect of AFC-based FSH dosage alterations, of which the results were described in two papers. An increased dose strategy in the expected poor responders did not result in higher pregnancy rates and was significantly more expensive [35], providing strong evidence to withdraw from high FSH dosing in this group. On the other hand, FSH dose reduction in the predicted hyper responders did not alter costs, yet did lower the overall rate of reported OHSS [36]. The occurrence of severe OHSS was not altered by FSH dose reduction. Thus, AFC-based dosing may be effective in improving safety, decreasing treatment costs, all without compromising the efficacy of the treatment. To note, in the OPTIMIST trial, agonist treatment protocols were studied. In addition to AFC-based dosing, one could also choose to use antagonist treatment protocols (in combination with agonist trigger)—a very effective strategy in decreasing the chance of developing OHSS [37].

The first study using AMH as an indicator for FSH dosage (in antagonist cycles) did show an increase in the ovarian response, but an effect on the cumulative ongoing pregnancy rate could not be found [38]. A few years later, the ESTHER-1 trial compared the efficacy and safety of individualized follitropin delta (rFSH) dosing (based on serum AMH and body weight) with conventional follitropin alfa dosing for ovarian stimulation in women undergoing first IVF cycles [39]. In the individualized dosing group, fewer poor responses and less excessive responses (and OHSS) were described, while maintaining pregnancy and implantation rate in a cost-effective manner (less gonadotropin use). Individualized tailored AMH-based dosing with rFSH compared to conventional dosing thus led to an improved safety without compromising efficacy in this trial. More recently, a Danish group conducted an RCT comparing individualized AMH-based dosing (either low, medium, or high AMH) with a standard group of 150 IU daily, irrespective of the AMH level [40]. Interestingly, the individualized (low) dosing of the predicted hyper responders (high AMH) resulted in significantly more poor responders, and the increased FSH dosing in the low AMH group resulted in an (almost 50%) reduction of poor responders. These changes however did not improve the cumulative live birth rate in the individualized dosing group (and was found to be comparable between the individualized and standard groups).

A meta-analysis of three phase 3 trials (including the GRAPE study) using Rekovelle (follitropin delta) was set up to investigate whether individualized-weight and AMH-based dosing improved live birth rate, safety, and efficiency as compared to standard FSH dosing. The study found that individualized dosing with Rekovelle was as effective in terms of live birth rate (with significantly higher live birth rates seen in normal to high responders) and reduced safety risks and FSH dosage compared to standard dosing in IVF [41].

In addition, a Cochrane analysis showed that ORT-based FSH dosing, compared to standard 150 IU daily dosing, reduces the incidence of moderate or severe OHSS. And also in this analysis, no increase in live birth rate was found in the ORT-based dosing group (pooled evidence of four studies [29]).

Together these studies indicate that ORT-based dosing helps us clinicians to lower the OHSS rate while maintaining pregnancy rates in a cost-effective manner. As mentioned earlier, ORTs do not predict pregnancy after ART and cannot be used for this objective. However, counselling on prognosis level based on age and AMH/AFC is interesting, although much of the information comes from female age, and adding tests could only be useful for counselling in certain subgroups, like older women (Table 38.1).

At the end of the tailoring treatment spectrum, when confronted with disappointing ORTs or ART outcome, couples could also be advised to apply for oocyte donation instead of undergoing another round of COS treatment (with expected poor response).

Reproductive lifespan prediction

Another challenge for ORTs lies in the identification of women with a reduced reproductive lifespan at such a stage in their lives that adequate action can be taken. Ideally, this could imply that these tests can be used to determine who will achieve a spontaneous pregnancy within a certain timeframe and who will need ART treatment. Also, and more realistically, such tests performed at a younger age could be used to predict the age at which a woman will become menopausal, since the relationship between menopausal age and the end of natural fertility has been hypothesized to be fixed (Figure 38.2) [7]. Therefore, based on reproductive lifespan forecasting, individualized preventive infertility management could become worthwhile.

Moreover, a woman's age at menopause is also related to various other general health issues. A late menopause age is associated with reduced all-cause morbidity and mortality, whereas women with an early menopause are at increased risk for osteoporosis, bone fractures, and cardiovascular risks. Therefore, prediction of menopause could not only be valuable regarding fertility but also for preventive strategies for general health [5].

Current fecundity prediction

In many Western countries, the average age of women giving birth to their first child is around 30 years. This means that a significant proportion of women when starting to try to conceive will already exhibit a reduced possibility of spontaneous pregnancy.

So far, three studies have been performed to assess the value of ovarian reserve testing in predicting spontaneous pregnancies. One study in 100 unselected women (aged 30–44 years) aiming to achieve a spontaneous pregnancy showed a good correlation between initial AMH levels and natural fertility in a six-month follow-up period [42]. However, these findings could not be confirmed in a second study, where no correlation was found between low AMH levels and reduced fecundability in women in their mid-twenties [43]. In a follow-up period up to 12 months, a third study showed that AMH levels did not predict time to ongoing pregnancy [44]. Therefore, to date, there is no role for ovarian reserve testing in the prediction of actual fecundity.

This is not surprising, as long as women have an ovulatory cycle the importance of the size of the cohort from which the follicle is selected is inferior to the quality of the dominant follicle.

Menopause prediction

As it is hypothesized that there is a fixed time interval between age at menopause and natural sterility, several studies have been undertaken regarding the role of ORTs in predicting menopause and thereby predicting age at natural sterility. If these tests were to be accurate, this may motivate some women to start a family at a younger age, or apply fertility-preservation techniques such as oocyte freezing. Alternatively, ovarian reserve testing could reassure others that postponing childbearing will not interfere with a woman's chances to achieve a pregnancy later on.

AMH has been studied more extensively in relation to menopause prediction. There are several studies consistently showing AMH to be associated with menopausal age, even after correction for age [45–51]. Age-specific AMH levels can be used to predict the age range in which menopause will occur (Figure 38.7 and Table 38.2). An IPD meta-analysis confirmed these findings.

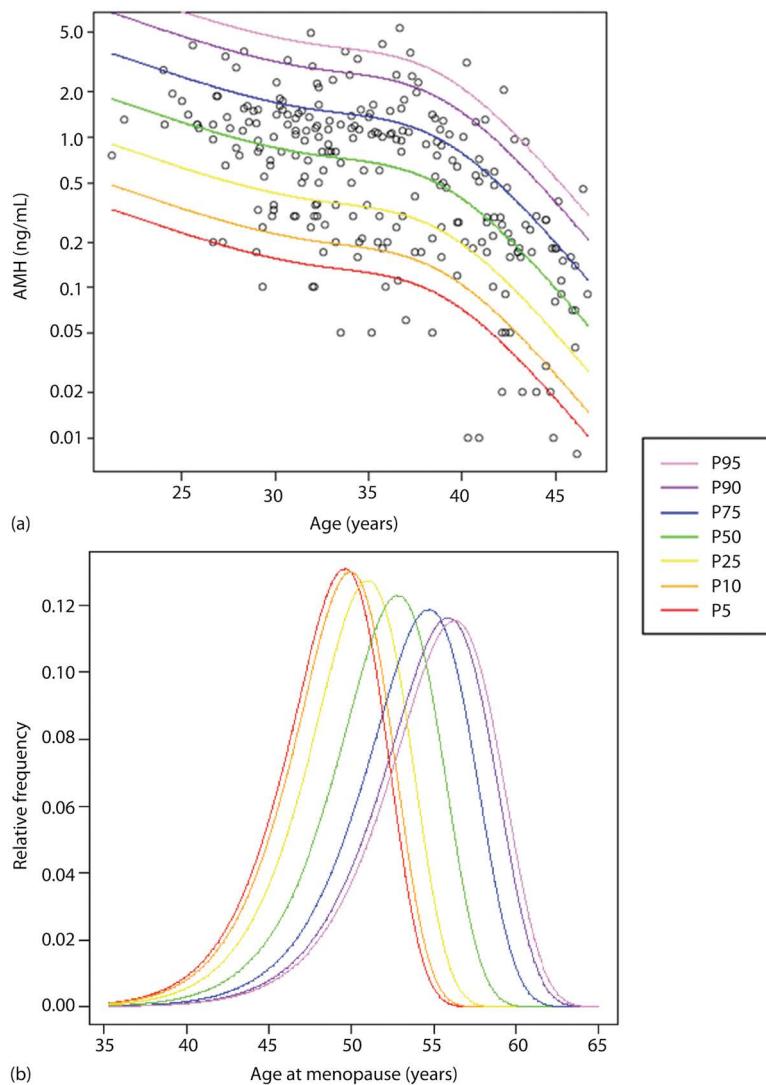


FIGURE 38.7 Nomograms for the relationship between age-specific AMH concentrations and the distribution of age at menopause. (a) The AMH levels measured at entry of the study for women at a given age are shown, measured approximately 11 years before cycle status assessment. The lines represent the upper margins of the different percentiles of AMH. Women can thus be placed in a percentile category based on their AMH concentration at a given age. (b) The variation of age at menopause for different percentiles of AMH. Abbreviation: AMH, anti-Mullerian hormone; P, percentile. (From [32], with permission.)

TABLE 38.2 Proportion of Women in Each AMH Percentile Category with Early, Normal, or Late Age at Menopause, as Derived from the Weibull Predicted Age at Menopause Distribution

Baseline AMH	Menopause \leq Age 45 y (9.6%)	Menopause Age 46–54 y (79.3%)	Menopause \geq Age 55 y (11.1%)
p5	28.1%	68.2%	3.8%
p10	25.3%	70.4%	4.3%
p25	17.7%	75.8%	6.5%
p50	8.0%	77.7%	14.2%
p75	2.7%	66.4%	30.9%
p90	1.2%	53.1%	45.6%
p95	0.9%	48%	51.1%

Source: From [52], with permission.

Note: The outer left column displays the age-specific AMH percentile categories. The top two rows display the age categories at which the event menopause occurred and their respective incidence. The incidence of the event menopause for each age category is displayed (irrespective of AMH levels). The seven bottom rows of the table display the distribution of age at menopause per AMH percentile category calculated using the Weibull model.

It also showed that the range of menopause prediction was limited in relation to the p-value AMH values (Table 38.2) [52].

However, the prediction models lack the capacity to predict the extreme ages of menopause (very young and very late) and have limited precision [50]. Specifically, these extreme ages at menopause are the most valuable to predict, as these have the main clinical value regarding the fertility lifespan and general health implications. With such limited precision, the clinical application of AMH testing to predict menopause becomes troublesome. One potential was to use repeated AMH measurements and thereby also get an indication of the decline of AMH over time. In a longitudinal analysis of five AMH measurements over 20 years, the speed of AMH decline was associated with AMH levels [53]. But the speed of decline did vary with age. And, at a younger age, the predictive accuracy of AMH was lower, thereby limiting the clinical utility of AMH as a predictor for menopause. Because exactly at a younger age, a woman would still have the possibility to change her reproductive plan. Accurate prediction at a later age, does not offer women these options.

Thus, although AMH is a very promising factor in the prediction of menopause, it is currently not applicable for predicting menopause or the end of natural fertility in day-to-day clinical practice.

Reproductive lifespan prediction in specific situations

Although assessment of the ovarian reserve in the general population has proven to be insufficient for clinical practice, there are

specific groups of women that may benefit from ovarian reserve testing, not only in the counselling of the expected reproductive lifespan but also in relation to long-term adverse health-related outcomes. Examples include the women at risk for early menopause—burdened by a family history of early menopause—or following gonadotoxic treatment.

For women at increased risk of premature ovarian insufficiency (POI), AMH may aid in the diagnosis; however, again it lacks the precision to accurately predict timing of onset of POI [54].

Interestingly, in a retrospective longitudinal follow-up of childhood cancer survivors it was shown that after initial impairment of the ovarian function, childhood cancer survivors follow a similar rate of decline in AMH over time compared to normal healthy controls [55].

Depending on the treatment received, childhood cancer survivors will be at risk for a reduced ovarian reserve and possible early menopause. Since AMH patterns post-treatment are similar to other women, ovarian reserve tests will be useful in assessing ovarian reserve in comparison to their age and may be useful in counselling on their reproductive lifespan, general health, and, if indicated, fertility preservation options [56].

Summary

Age-related fertility decline varies considerably among women. Therefore, chronological female age, though informative for pregnancy prospects in assisted reproduction, will not always correctly express a woman's reproductive potential. Currently, ORTs have

TABLE 38.3 Characteristics of the Studies Included in the Meta-Analysis regarding Anti-Mullerian Hormone and Prediction of Menopause

First Author (Year)	Patients (n)	MP at FU (n)	Outcome Variable	Analytical Method	Results		
					HR	C-statistic	Other
Sowers (2010)	50	50	TTM	Generalized estimating equations and mixed model analysis	—	—	\log_{10} AMH 1 unit lower, age of menopause 1.75 years earlier (± 0.14)
Tehrani (2011)	266	63	ANM	Accelerated failure time modelling and AUC	—	—	Acceptable agreement between observed and predicted ANM (bias -0.3 ; 95% CI -4 to 3 years); individual ANM predictions
Broer (2011)	281	48	TTM and ANM	Cox regression analysis + C-statistic	HR 9.2; 95% CI 2.5–34; $p < 0.001$ 1 unit AMH = 0.89 ng/mL	90%	—
Freeman (2012)	401	198	TTM	Cox regression analysis	HR 5.6; 95% CI 4.7–6.7; $p < 0.0001$ 1 unit = $1 SD_{\log_{10} \text{AMH}}$	—	—
Tehrani (2013)	1015	277	ANM	Accelerated failure time modelling and AUC + C-statistic	—	92%	Good agreement between observed and predicted ANM; individual ANM predictions
Dölleman (2015)	1163	527	TTM	Cox regression analysis + C-statistic	HR 9.1; SD 0.03	89%	—
Nair (2015)	716	207	TTM	Discrete time hazard regression in three-year intervals from baseline	HR 0–3 years: 8.1 HR 3–6 years: 2.3 HR 6–9 years: 1.6	—	—

Source: From [50], with permission.

Abbreviations: AMH, anti-Mullerian hormone; AUC, area under the curve; MP, reached menopause; FU, follow up; ANM, age at natural menopause; TTM, time to menopause; HR, hazard ratio (percentage increase in chance of MP occurring during FU per [unit] decrease of AMH); CI, confidence interval; C-statistic, percentage of correctly predicted MP occurring during FU.

been shown to be accurate predictors of the quantitative aspects of the ovarian reserve and thereby of the response to COS and may aid in choosing ovarian stimulation protocol. However, they are not accurate predictors of the qualitative aspect of the ovarian reserve and thus are not good predictors of pregnancy after IVF.

For the prediction of the reproductive lifespan, mainly AMH has been studied (Table 38.3). AMH is not applicable for the prediction of fecundity. For the prediction of menopause and thereby the end of natural fertility, there is consistent evidence that AMH is a good predictor; however, due to wide prediction intervals, AMH is currently not applicable in day-to-day clinical practice for the general population for these purposes. Only specific subgroups of women with an increased risk of early menopause might benefit of such measurements.

References

- Stephen EH, Chandra A. Declining estimates of infertility in the United States: 1982–2002. *Fertil Steril*. 2006;86:516–23.
- Te Velde ER, Pearson PL. The variability of female reproductive aging. *Hum Reprod Update*. 2002;8:141–54.
- Morabia A, Costanza MC. International variability in ages at menarche, first livebirth, and menopause. World Health Organization collaborative study of neoplasia and steroid contraceptives. *Am J Epidemiol*. 1998;148:1195–205.
- Thomas F, Renaud F, Benefice E, de Meeus T, Guegan JF. International variability of ages at menarche and menopause: Patterns and main determinants. *Hum Biol*. 2001;73:271–90.
- Daan NM, Fauser BC. Menopause prediction and potential implications. *Maturitas*. 2015;82:257–65.
- van Noord-Zaadstra BM, Loosman CWN, Alsbach H, et al. Delaying childbearing: Effect of age on fecundity and outcome of pregnancy. *BMJ*. 1991;302:1361–5.
- Broekmans FJ, Soules MR, Fauser BC. Ovarian aging: Mechanisms and clinical consequences. *Endocr Rev*. 2009;30:465–93.
- Hamdine O, Eijkemans MJ, Lentjes EW, Torrance HL, Macklon NS, Fauser BC, Broekmans FJ. Antimüllerian hormone: Prediction of cumulative live birth in gonadotropin-releasing hormone antagonist treatment for *in vitro* fertilization. *Fertil Steril*. 2015;104: 891–8.
- Bruin JP, te Velde ER. Female reproductive aging: Concepts and consequences. In: *Preservation of Fertility*. Tulandi T, Gosden RG (eds.), p. 3. London: Taylor & Francis, 2004.
- Viudes-de-Castro MP, Vicente JS. Effect of sperm count on the fertility and prolificacy rates of meat rabbits. *Anim Reprod Sci*. 1997;46:313–9.
- Moce E, Lavara R, Vicente JS. Influence of the donor male on the fertility of frozen–thawed rabbit sperm after artificial insemination of females of different genotypes. *Reprod Domest Anim*. 2005;40:516–21.
- Evers JL. Female subfertility. *Lancet*. 2002;360:151–9.
- Menken J, Trussell J, Larsen U. Age and infertility. *Science*. 1986;233:1389–94.
- Oudendijk JF, Yarde F, Eijkemans MJ, Broekmans FJ, Broer SL. The poor responder in IVF: Is the prognosis always poor? A systematic review. *Hum Reprod Update*. 2012;18:1–11.
- Templeton A, Morris JK, Parslow W. Factors that affect outcome of *in-vitro* fertilisation treatment. *Lancet*. 1996;348:1402–6.
- Tarlatzis BC, Zepiridis L, Grimbizis G, Bontis J. Clinical management of low ovarian response to stimulation for IVF: A systematic review. *Hum Reprod Update*. 2003;9:61–76.
- Hendriks DJ, Mol BW, Bancsi LF, te Velde ER, Broekmans FJ. Antral follicle count in the prediction of poor ovarian response and pregnancy after *in vitro* fertilization: A metaanalysis and comparison with basal follicle-stimulating hormone level. *Fertil Steril*. 2005;83:291–301.
- Broekmans FJ, Kwee J, Hendriks DJ, Mol BW, Lambalk CB. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update*. 2006;12:685–718.
- Seifer DB, MacLaughlin DT, Christian BP, Feng B, Shelden RM. Early follicular serum mullerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technique cycles. *Fertil Steril*. 2002;77:468–71.
- Klein NA, Houmard BS, Hansen KR, et al. Age-related analysis of inhibin A, inhibin B, and activin A relative to the intercycle monotropic follicle-stimulating hormone rise in normal ovulatory women. *J Clin Endocrinol Metab*. 2004;89:2977–81.
- Soules MR, Battaglia DE, Klein NA. Inhibin and reproductive aging in women. *Maturitas*. 1998; 30(2):193–204.
- Fasouliotis SJ, Simon A, Laufer N. Evaluation and treatment of low responders in assisted reproductive technique: A challenge to meet. *J Assist Reprod Genet*. 2000;17:357–73.
- Popovic-Todorovic B, Loft A, Lindhard A, et al. A prospective study of predictive factors of ovarian response in “standard” IVF/ICSI patients treated with recombinant FSH. A suggestion for a recombinant FSH dosage nomogram. *Hum Reprod*. 2003;18:781–7.
- van der Gaast MH, Eijkemans MJ, van der Net JB, et al. Optimum number of oocytes for a successful first IVF treatment cycle. *Reprod Biomed Online*. 2006;13:476–80.
- Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L, ESHRE working group on Poor Ovarian Response Definition. ESHRE consensus on the definition of “poor response” to ovarian stimulation for *in vitro* fertilization: The Bologna criteria. *Hum Reprod*. 2011;26:1616–24.
- Broer SL, van Disseldorp J, Broeze KA, Dolleman M, Opmeer BC, Bossuyt P, Eijkemans MJ, Mol BW, Broekmans FJ, IMPORT Study Group. Added value of ovarian reserve testing on patient characteristics in the prediction of ovarian response and ongoing pregnancy: An individual patient data approach. *Hum Reprod Update*. 2013;19:26–36.
- Lashen H, Ledger W, Lopez-Bernal A, Barlow D. Poor responders to ovulation induction: Is proceeding to *in-vitro* fertilization worthwhile? *Hum Reprod*. 1999;14:964–9.
- Verberg MF, Eijkemans MJ, Macklon NS, et al. Predictors of low response to mild ovarian stimulation initiated on cycle day 5 for IVF. *Hum Reprod*. 2007;22:1919–24.
- Lensen SF, Wilkinson J, Leijdekkers JA, La Marca A, Mol BWJ, Marjoribanks J, Torrance H, Broekmans FJ. Individualised gonadotropin dose selection using markers of ovarian reserve for women undergoing *in vitro* fertilisation plus intracytoplasmic sperm injection (IVF/ICSI). *Cochrane Database Syst Rev*. 2018;2: CD012693.
- Fauser BC, Diedrich K, Devroey P. Predictors of ovarian response: Progress towards individualized treatment in ovulation induction and ovarian stimulation. *Hum Reprod Update*. 2008; 14:1–14.
- Sunkara SK, Rittenberg V, Raine-Fenning N, et al. Association between the number of eggs and live birth in IVF treatment: An analysis of 400 135 treatment cycles. *Hum Reprod*. 2011;26:1768–74.
- Broer SL, Dolleman M, van Disseldorp J, Broeze KA, Opmeer BC, Bossuyt PM, Eijkemans MJ, Mol BW, Broekmans FJ, IPD-EXPORT Study Group. Prediction of an excessive response in *in vitro* fertilization from patient characteristics and ovarian reserve tests and comparison in subgroups: An individual patient data meta-analysis. *Fertil Steril*. 2013;100:420–9.
- La Marca A, Nelson SM, Siginolfi G, et al. Anti-mullerian hormone-based prediction model for a live birth in assisted reproduction. *Reprod Biomed Online*. 2011;22:341–9.
- Klinkert ER, Broekmans FJ, Loosman CW, Habbema JD, Te Velde ER. Expected poor responders on the basis of an antral follicle count do not benefit from a higher starting dose of gonadotrophins in IVF treatment: A randomized controlled trial. *Hum Reprod*. 2005;20:611–15.

35. van Tilborg TC, Torrance HL, Oudshoorn SC, Eijkemans MJC, Koks CAM, Verhoeve HR, Nap AW, Scheffer GJ, Manger AP, Schoot BC, et al. Individualized versus standard FSH dosing in women starting IVF/ICSI: An RCT. Part 1: The predicted poor responder. *Hum Reprod.* 2017;32:2496–505.
36. Oudshoorn SC, van Tilborg TC, Eijkemans MJC, Oosterhuis GJE, Friederich J, van Hooff MHA, van Santbrink EJP, Brinkhuis EA, Smeenk JM, Kwee J, et al. Individualized versus standard FSH dosing in women starting IVF/ICSI: An RCT. Part 2: The predicted hyper responder. *Hum Reprod.* 2017;32:2506–14.
37. Mourad S, Brown J, Farquhar C. Interventions for the prevention of OHSS in ART cycles: An overview of Cochrane reviews. *Cochrane Database Syst Rev.* 2017; Jan 23; 1(1):CD012103.
38. Arce JC, Andersen AN, Fernández-Sánchez M, Visnova H, Bosch E, García-Velasco JA, Barri P, de Sutter P, Klein BM, Fauser BC. Ovarian response to recombinant human follicle-stimulating hormone: A randomized, antimüllerian hormone-stratified, dose-response trial in women undergoing in vitro fertilization/intracytoplasmic sperm injection. *Fertil Steril.* 2014;102:1633–40.
39. Nyboe Andersen A, Nelson SM, Fauser BCJM, García-Velasco JA, Klein BM, Arce J-C, ESTHER-1 Study Group, Tournaye H, De Sutter P, Decleer W, Petracco A, et al. Individualized versus conventional ovarian stimulation for in vitro fertilization: A multicenter, randomized, controlled, assessor-blinded, phase 3 noninferiority trial. *Fertil Steril.* 2017;107:387–96.
40. Petersen FJ, Løkkegaard E, Andersen LF, Torp K, Egeberg A, Hedegaard L, Nysom D, Nyboe Andersen A. A randomized controlled trial of AMH-based individualized FSH dosing in a GnRH antagonist protocol for IVF. *Hum Reprod Open.* 2019;(1):hoz003.
41. Janse F, Eijkemans M, Fauser B. O-114 improved safety and efficiency of individualised versus conventional gonadotropin dosing for ovarian stimulation in IVF/ICSI: An individual patient meta-Analysis (IPD-MA). *Hum Reprod.* 2021; 36(Suppl 1):deab126.023
42. Steiner AZ, Herring AH, Kesner JS, Meadows JW, Stanczyk FZ, Hoberman S, Baird DD. Antimüllerian hormone as a predictor of natural fecundability in women aged 30–42 years. *Obstet Gynecol.* 2011;117:798–804.
43. Hagen CP, Vestergaard S, Juul A, et al. Low concentration of circulating antimüllerian hormone is not predictive of reduced fecundability in young healthy women: A prospective cohort study. *Fertil Steril.* 2012;98:1602–8.
44. Depmann M, Broer SL, Eijkemans MJC, van Rooij IAJ, Scheffer GJ, Heimensem J, Mol BW, Broekmans FJM. Anti-müllerian hormone does not predict time to pregnancy: Results of a prospective cohort study. *Gynecol Endocrinol.* 2017;10:1–5.
45. Broer SL, Eijkemans MJ, Scheffer GJ, et al. Antimüllerian hormone predicts menopause: A long-term follow-up study in normoovulatory women. *J Clin Endocrinol Metab.* 2011;96:2532–9.
46. Tehrani FR, Shakeri N, Solaymani-Dodaran M, Azizi F. Predicting age at menopause from serum antimüllerian hormone concentration. *Menopause.* 2011;18:766–70.
47. Nair S, Slaughter JC, Terry JG, et al. Anti-müllerian hormone (AMH) is associated with natural menopause in a population-based sample: The CARDIA Women's Study. *Maturitas.* 2015;81:493–8.
48. Dölleman M, Verschuren WM, Eijkemans MJ, Broekmans FJ, van der Schouw YT. Added value of anti-müllerian hormone in prediction of menopause: Results from a large prospective cohort study. *Hum Reprod.* 2015;30:1974–81.
49. Dölleman M, Depmann M, Eijkemans MJ, et al. Anti-müllerian hormone is a more accurate predictor of individual time to menopause than mother's age at menopause. *Hum Reprod.* 2014;29:584–91.
50. Depmann M, Broer SL, van der Schouw YT, Tehrani FR, Eijkemans MJ, Mol BW, Broekmans FJ. Can we predict age at natural menopause using ovarian reserve tests or mother's age at menopause? A systematic literature review. *Menopause.* 2016;23:224–32.
51. Depmann M, Eijkemans MJC, Broer SL, Scheffer GJ, van Rooij IAJ, Laven JSE, Broekmans FJM. Does AMH predict menopause? Results of a prospective ongoing cohort study. *Hum Reprod.* 2016;31:1579–87.
52. Depmann M, Eijkemans MJC, Broer SL, Tehrani FR, Solaymani-Dodaran M, Azizi F, Lambalk CB, Randolph JF Jr., Harlow SB, Freeman EW, Sammel MD, Verschuren M, van der Schouw YT, Mol BW, Broekmans FJM. Does AMH relate to timing of menopause? Results of an individual patient data meta-analysis. *J Clin Endocrinol Metabol.* 2018;103(10):3593–600.
53. de Kat AC, van der Schouw YT, Eijkemans MJC, Broer SL, Verschuren M, Broekmans FJM. Can menopause prediction be improved with multiple AMH measurements? Results from the prospective Doetinchem cohort study. *J Clin Endocrinol Metabol.* 2019;104(11):5024–31.
54. Anderson RA, Nelson SM. Anti-müllerian hormone in the diagnosis and prediction of premature ovarian insufficiency. *Semin Reprod Med.* 2020;38:263–69.
55. van der Kooi AL, van den Heuvel-Eibrink MM, van Noortwijk A, Neggers SJ, Pluijm SM, van Dulmen-den Broeder E, van Dorp W, Laven JS. Longitudinal follow-up in female childhood cancer survivors: No signs of accelerated ovarian function loss. *Hum Reprod.* 2017;32(1):193–200.
56. van den Berg MH, Overbeek A, Lambalk CB, Kaspers GJL, Bresters D, van den Heuvel-Eibrink MM, Kremer LC, Loonen JJ, van der Pal HJ, Ronckers CM, Tissing WJE, Versluys AB, van der Heiden-van der Loo M, Heijboer AC, Hauptmann M, Twisk JWR, Laven JSE, Beerendonk CCM, van Leeuwen FE, van Dulmen-den Broeder E, DCOG LATER-VEVO study group. Long-term effects of childhood cancer treatment on hormonal and ultrasound markers of ovarian reserve. *Hum Reprod.* 2018;33(8):1474–88.

DRUGS USED FOR OVARIAN STIMULATION

Clomiphene Citrate, Aromatase Inhibitors, Metformin, Gonadotropin-Releasing Hormone Analogues, and Gonadotropins

Colin M. Howles and Zeev Shoham

Introduction

Infertility treatment became available owing to developments in the characterization and purification of hormones. Treatment with urinary-derived human gonadotropins and clomiphene citrate (CC) became available in 1961, and then over the following 35 years advancements in production techniques, including the use of recombinant DNA technology [1], led to the availability of purer and more consistent injectable gonadotropins (for a review, see [2]). The purpose of this chapter is to overview the development, structure, and mode of action of treatments for ovulation induction (OI) and controlled ovarian stimulation (COS) for assisted reproduction technologies (ARTs).

Clomiphene citrate

Drug description

CC was synthesized in 1956, and an indisputable therapeutic breakthrough occurred in 1961 when Greenblatt and his group discovered that CC, a nonsteroidal analogue of oestradiol, exerts a stimulatory effect on ovarian function in women with anovulatory infertility [3]. The drug was approved for infertility treatment by the US Food and Drug Administration in 1967.

CC is a triphenylchloroethylene derivative in which the four hydrogen atoms of the ethylene core have been substituted with three phenyl rings and a chloride anion. One of the three phenyl rings bears an aminoalkoxy ($\text{OCH}_2\text{--CH}_2\text{--N}[\text{C}_2\text{K}_2]$) side chain, but the importance of its action on CC remains uncertain. The dihydrogen citrate moiety ($\text{C}_6\text{H}_8\text{O}_7$) accounts for the fact that commercially available preparations represent the dihydrogen citrate salt form of CC. CC is a white or pale-yellow odourless powder, unstable in air and light, with a melting point of 116–118°C. It is a triarylethylene compound (1-*p*-diethyl aminoethoxyphenyl-1,2-diphenyl-2-chloroethylene citrate, with a molecular weight of 598.09) that is chemically related to chlorotrianisene, which is a weak oestrogen. Structurally, CC is related to diethylstilbestrol, a potent synthetic oestrogen. Although this compound is not a steroid, but a triphenylchloroethylene, its configuration bears a remarkable structural similarity to oestradiol, and consequently facilitates binding to oestrogen receptors (ERs).

CC is available as a racemic mixture of two stereochemical isomers referred to as (*cis*) Zu-clomiphene or the (*trans*) En-clomiphene configuration (Figure 39.1a, b), the former being significantly more potent. In the commercially available preparations, the isomers are in the ratio of 38% Zu-clomiphene and 62% En-clomiphene. Limited experience suggests that the clinical utility of CC may indeed be due to its *cis* isomer [4, 5]. However, it remains uncertain whether *cis*-CC is more effective than CC proper in terms of ovulation and conception rates [6–9].

Following the development of a reverse-phase high performance liquid chromatography (HPLC) assay [10], it was apparent that each isomer exhibited its own characteristic pharmacokinetic profile, the En isomer being absorbed faster and eliminated more completely than the Zu isomer. Although CC tablets contain 62% En isomer and 38% Zu isomer, the observed plasma concentrations of the Zu isomer were much higher than those of the En isomer. Because the Zu isomer is considered more oestrogenic than the En isomer, response of the target tissues should vary according to both the relative affinity and the concentrations of each isomer interacting with the relevant ER. Tracer studies of CC with radioactive carbon labelling have shown that the main route of excretion is via the faeces, although small amounts are also excreted in the urine. After administration of CC for five consecutive days at a dose of 100 mg daily, the drug could be detected in serum for up to 30 days.

Mechanism of action

Administration of CC is followed in short sequence by enhanced release of pituitary gonadotropins, resulting in follicular recruitment, selection, assertion of dominance, and rupture.

The principal mechanism of CC action is a reduction in the negative feedback of endogenous oestrogens due to prolonged depletion of hypothalamic and pituitary ERs [11, 12]. This action consequently leads to an increase in the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus into the hypothalamic–pituitary portal circulation, engendering an increase in the release of pituitary gonadotropins. Administration of a moderate gonadotropin stimulus to the ovary overcomes the ovulation disturbances and increases the cohort of follicles reaching ovulation [13, 14]. A marked increase in serum concentrations of luteinizing hormone (LH) in proportion to follicle-stimulating hormone (FSH) may sometimes occur [15], and this temporary change in the LH:FSH ratio appears to bring about some impairment of follicular maturation, resulting in delayed ovulation. Shortly after discontinuation of CC, both gonadotropins gradually decline to the pre-ovulatory nadir, only to surge again at mid-cycle.

The drug interacts with ER-binding proteins similar to native oestrogens and behaves as a competitive ER antagonist [16, 17]. Importantly, CC does not display progestational, corticotropic, androgenic, or antiandrogenic properties.

Indications and contraindications for treatment

Anovulatory infertility is the most important indication for CC treatment. In addition, treatment is indicated for women with oligomenorrhoea, or amenorrhoea, who responded to progesterone (P) treatment with withdrawal bleeding. Treatment is ineffective in women with hypogonadotropic hypogonadism (HH; World Health Organization [WHO] group I). Other controversial

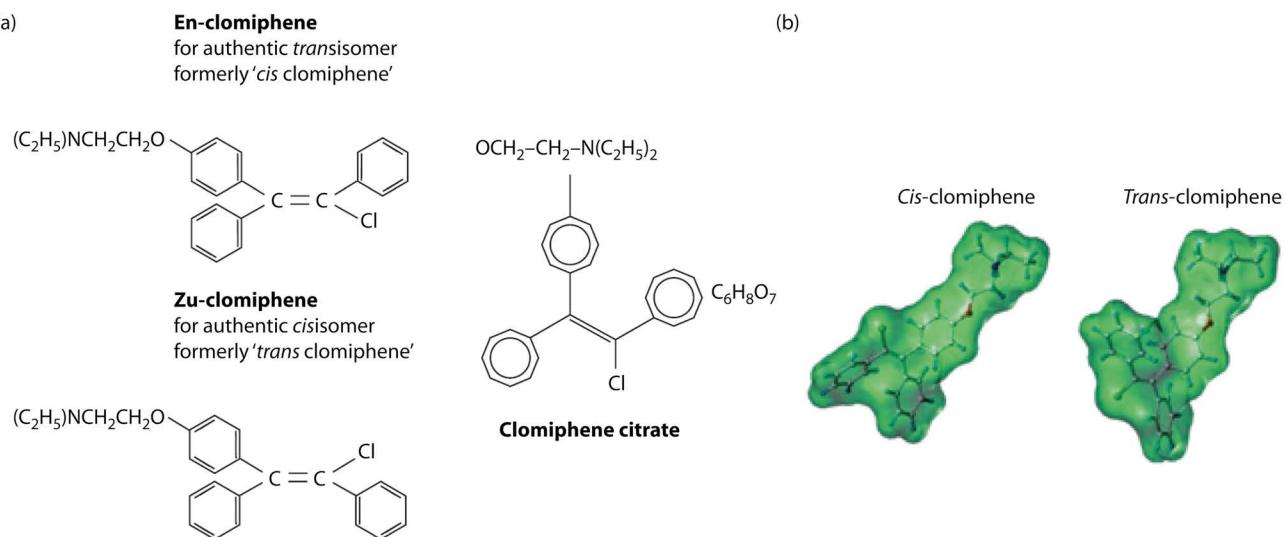


FIGURE 39.1 (a) Clomiphene citrate is available as a racemic mixture of two stereochemical isomers referred to as (*cis*) Zu-clomiphene or the (*trans*) En-clomiphene configuration, with the former being significantly more potent. In the preparations that are commercially available, the isomers are in a ratio of 38% Zu-clomiphene and 62% En-clomiphene. (b) The isomeric models in a different configuration.

indications include luteal-phase defect, unexplained infertility, and women undergoing *in vitro* fertilization (IVF) when multiple follicle development is required. Contraindications to CC administration include pre-existing ovarian cysts, with suspected malignancy, and liver disease.

Duration of treatment

CC increases secretion of FSH and LH and is administered for a period of five days. In women with normal cycles, administration of CC for more than five days resulted in an initial increase of serum FSH concentration that lasted for five to six days, followed by a decline in serum FSH levels, despite continuation of the drug, whereas LH levels remained high throughout the entire treatment period [18, 19].

CC is usually administered on day 5 of spontaneous or induced menstruation. This is based on the theory that on day 5 the physiologic decrease in serum FSH concentration provides the means for selection of the dominant follicle. Initiation of the drug on day 2 induces earlier ovulation, which is analogous to the physiologic events of the normal menstrual cycle. The starting dose is usually 50 mg/day, owing to the observation that 50% of pregnancies occur with the 50-mg dose [20]. In order to obtain good results, CC therapy should be carefully monitored. Obviously, serial measurements of LH, FSH, oestradiol, and P and ultrasound measurements provide the most detailed information on the patient's response to treatment.

Results of treatment

CC induces ovulation in the majority of women. The ovulation rate ranges between 70% and 92%; however, the pregnancy rate is much lower. The discrepancy between the high ovulation rates and relatively low pregnancy rates may be due to the following factors: (1) antioestrogen effects on the endometrium; (2) anti-oestrogen effects on the cervical mucus; (3) decrease of uterine blood flow; (4) impaired placental protein 14 synthesis; (5) sub-clinical pregnancy loss; (6) effect on tubal transport; and (7) detrimental effects on the oocytes [21]. The Cochrane review [22] of

clinical data regarding the use of CC for unexplained sub-fertility in women, based on five randomized trials of CC (doses ranging from 50 to 250 mg/day for up to 10 days) compared with placebo or no treatment, showed that the odds ratio (OR) for pregnancy per patient was 2.38 (95% confidence interval [CI] 1.22–4.62). The OR for pregnancy per cycle was 2.5 (95% CI 1.35–4.62). It was concluded from this review that CC appeared to improve pregnancy rates modestly in women with unexplained sub-fertility.

Side effects and safety

The most common side effects are hot flushes (10%); abdominal distension, bloating, or discomfort (5%); breast discomfort (2%); nausea and vomiting (2%); visual symptoms and headache (1.5%). A rise in basal body temperature may be noted during the five-day period of CC administration. Visual symptoms include spots (floaters), flashes, or abnormal perception. These symptoms are rare, universally disappear upon cessation of CC therapy, and have no permanent effect. The multiple pregnancy rate is approximately 5% and almost exclusively due to twins.

Several reports have associated long-term (>12 months) CC therapy with a slight increase in future risk of ovarian cancer (relative risk [RR] = 1.5–2.5) [23]. Owing to these initial reports, the Committee on Safety of Medicines in the United Kingdom advised doctors to adhere to the manufacturers' recommendations of limiting treatment to a maximum of six months. However, this increased risk has not been confirmed by subsequent reports. Several case reports have linked CC with congenital malformations, especially neural tube defects [24–30]. Data available on 3751 births after CC treatment included 122 children born with congenital malformations (major and minor), representing an incidence of 32.5/1000 births [31]. This figure is within the range found among the normal population [32].

Summary

CC is one of the most popular drugs for OI because it is easy to administer, highly effective, considered safe, and the cost is minimal.

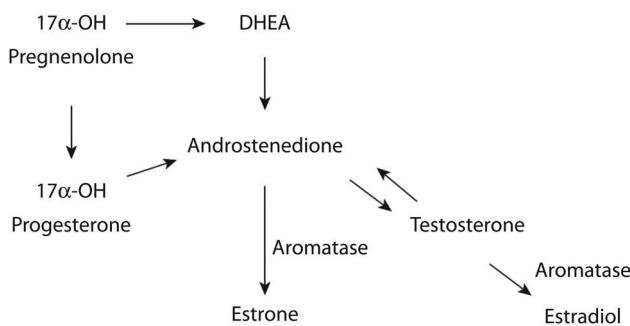


FIGURE 39.2 Aromatase inhibitor. Aromatase, an enzyme found in the liver, is responsible for the conversion of androgens—*androstenedione* and *testosterone*—into oestrogens—*oestrone* and *oestradiol*. By inhibiting aromatase, the body produces less oestrogen and maintains a higher testosterone state. Abbreviation: DHEA, dehydroepiandrosterone sulphate.

Aromatase inhibitors

Aromatase, a cytochrome P450-dependent enzyme, acts as the ultimate step in the synthesis of oestrogen, catalyzing the conversion of androgens to oestrogens [33]. The conversion of androgens to oestrogens also occurs at peripheral sites, such as in muscle, fat, and the liver [34]. Recently, a group of new, highly selective aromatase inhibitors has been approved to suppress oestrogen production in postmenopausal women with breast cancer. Aromatase inhibitor is a competitive inhibitor of the aromatase enzyme system and inhibits the conversion of androgens to oestrogens. It inhibits the aromatase enzyme by competitively binding to the heme of the aromatase–cytochrome P450 subunit of the enzyme, resulting in a reduction of oestrogen biosynthesis in all tissues where it is present (Figure 39.2). Treatment significantly lowers serum estrone, oestradiol, and estrone sulphate, and has not been shown significantly to affect adrenal corticosteroid synthesis, aldosterone synthesis, or synthesis of thyroid hormones. Maximum suppression is achieved within 48–78 hours. The first aromatase inhibitor to be developed was aminoglutethimide, but its usage was stopped owing to side effects, one of which was adrenal insufficiency [35]. However, this development stimulated the formulation of numerous other aromatase inhibitors that were described as first-, second-, and third-generation inhibitors according to chronologic development. They were further classified as type I (steroid analogues of *androstenedione*) and type II (nonsteroidal) (Table 39.1).

TABLE 39.1 Different Types and Generations of Aromatase Inhibitors

Generation	Type I	Type II
First	None	Aminoglutethimide
Second	Formestane	Fadrozole Rogletimide
Third	Exemestane	Anastrozole Letrozole Vorozole

Pharmacokinetics

Third-generation aromatase inhibitors are administered orally, and have a half-life of approximately 48 hours, which allows once-daily dosing [36, 37]. These drugs metabolize mainly in the liver, and are excreted through the biliary (85%) and the urinary (11%) systems.

Side effects and safety

Reported side effects are bone pain (20%), hot flushes (18%), back pain (17%), nausea (15%), and dyspnoea (14%). These side effects are typically observed after long-term administration.

One major concern is the use of letrozole in OI or COS because of its possible teratogenicity as observed in animal models. There was one concerning report (published as an abstract only) [38] of an increase in cardiac and bone malformations in letrozole-treated pregnancies. Following the publication of the abstract, the manufacturer, Novartis, wrote to clinicians in the United States and Canada stating that letrozole was not safe for use in women who were either desiring pregnancy or pregnant. Since this notification, there has been a series of published studies, including a multicentre retrospective analysis of 911 new-borns conceived after CC or letrozole treatment [39]. This did not show any teratogenic effect of letrozole, and they reported a similar rate of congenital malformation to that seen in women conceiving after treatment with CC. In the most recent paper from Badawy et al. [40], they also stated that there were no observed increases in congenital malformations following the use of letrozole. Subsequently, two large prospective randomized trials have studied letrozole in polycystic ovary syndrome (PCOS) [41] and unexplained infertility [42]. They have shown that cumulative rates of teratogenicity with letrozole are <5% and comparable to rates with clomiphene. These results are reassuring and have led one recent reviewer to ask not necessarily for more safety data, but rather for evidence of any harm as manifested by higher rates of congenital abnormalities [43].

Drugs available

Letrozole: this is chemically described as 4,4'-(1*H*-1,2,4-triazole-1-ylmethylene) dibenzonitrile, with a molecular weight of 285.31 and an empirical formula of $C_{17}H_{11}N_5$.

Anastrozole: the molecular formula is $C_{17}H_{19}N_5$ and it has a molecular weight of 293.4.

Both drugs are approved for the treatment of breast cancer in postmenopausal women.

The first clinical study using an aromatase inhibitor (letrozole: Novartis) for OI was published by Mitwally and Casper in 2001 [44]. With letrozole treatment in patients with PCOS, ovulation occurred in 75% and pregnancy was achieved in 25%. Letrozole appears to prevent unfavourable effects on the endometrium that are frequently observed with anti-oestrogen use for OI. Since the initial observation, several studies have been published on the use of aromatase inhibitors in the treatment of infertile patients [45–47]. The same investigators [48] showed that the use of an aromatase inhibitor reduced the FSH dose required for ovarian stimulation, without the undesirable anti-oestrogenic effects occasionally noted with CC.

It is now 23 years since the first successful report of the use of aromatase inhibitors in OI. There have been numerous studies over the intervening years and meta-analyses. For instance, a meta-analysis of six randomized controlled trials (RCTs) involving 841 patients with PCOS showed no significant differences in pregnancy, abortion, or multiple pregnancy rates between CC

and letrozole [49]. The authors concluded that letrozole may be as effective as CC for OI in patients with PCOS [49]. In the most updated Cochrane Systematic review [50], Franik et al. reviewed 42 RCTs (7935 women), and letrozole was used in all studies. The authors concluded that letrozole, vs CC, appeared to improve live birth and pregnancy rates in women with PCOS. There was high-quality evidence of no difference in miscarriage or multiple pregnancy rates, and OHSS rates were similar with letrozole vs CC. There is, however, still the need to carry out further trials to investigate different dosing regimens (e.g. 5- vs 10-day course of administration), however caution is required due to the potential concerns of teratogenic effects of letrozole, thus follow-up is required in terms of outcomes on neonatal birth defects.

Two randomized studies have also compared the efficacy and safety of single-dose and multi-dose anastrozole with CC in infertile women with ovulatory dysfunction [51, 52]. Anastrozole was found to be less effective than CC at inducing ovulation in both studies. Anastrozole has also been shown to have a weaker effect on follicular growth than CC [53].

Aromatase inhibitors have also been investigated for use in ART. Four randomized trials have been published with letrozole in a total of 235 patients with poor ovarian response [54–57]. When letrozole was combined with FSH, the gonadotropin dose required was consistently lower than when gonadotropins were used alone. In three trials, pregnancy rates were comparable in the treatment arms [55–57], and in one trial, pregnancy rates were lower in the letrozole arm than in the control arm [54]. Only one randomized trial with letrozole has been reported in patients with normal ovarian response undergoing IVF or intracytoplasmic sperm injection (ICSI) [58]. This was a pilot study involving 20 patients and showed an increased number of oocytes retrieved, and increased implantation and clinical pregnancy rates when letrozole was added to recombinant human follicle stimulating hormone (r-hFSH) [58]. However, no significant difference between groups was shown, possibly owing to the small study population.

More recently, there has been a systematic review and meta-analysis [59] published on letrozole co-treatment during OS in both normo and poor responders for ART. Thirty-one studies were included (20 investigating poor responders) and it was found that the live birth rate (LBR) in poor responders was significantly increased, by 7% ($P = 0.03$), with letrozole co-treatment. Gonadotrophin consumption was also significantly reduced, without decreasing the number of retrieved oocytes. However, in normal responders, number of oocytes was increased ($P = 0.01$) with letrozole co-treatment, but there was not a significant effect on LBR. The authors concluded that the effect of letrozole suppressing oestradiol levels at day of triggering follicular maturation was most consistently achieved using an antagonist protocol with 5-mg letrozole a day for a minimum of five days.

Metformin

The biguanide metformin (dimethylbiguanide) is an oral antihyperglycaemic agent widely used in the management of non-insulin-dependent diabetes mellitus. It is an insulin sensitizer that reduces insulin resistance and insulin secretion. Over the last few years there has been increased interest in the use of metformin (at doses of 1500–2500 mg/day) to increase ovulatory frequency, particularly in women described as having PCOS.

There is, however, some recent conflicting evidence regarding the usefulness of metformin in PCOS patients. In a Cochrane

systematic review [60], metformin was concluded to be an effective treatment for anovulation in women with PCOS, with it being recommended to be a first-line treatment, and with some evidence of benefit on parameters of the metabolic syndrome. Ovulation rates were higher when combined with clomiphene (76% vs 46% when used alone). Finally, the authors recommended that it should be used as an adjuvant to general lifestyle improvements, and not as a replacement for increased exercise and improved diet.

Subsequently, Lobo [61] and the National Institute for Health and Care Excellence (United Kingdom) [62] have made recommendations for its use in treating anovulatory PCOS. In previously untreated women with PCOS, no superiority of the combination of CC and metformin, rather than CC alone, was demonstrated in a large, Dutch multicentre study [63]. In a “head-to-head” study comparing CC with metformin as first-line treatment, although ovulation and pregnancy rates were similar, significantly fewer miscarriages and, therefore, more live births were achieved with metformin [64]. In a meta-analysis of randomized trials in PCOS patients undergoing OI or IVF/embryo transfer (ET) [65], co-administration of metformin with gonadotropins did not significantly improve ovulation (OR = 3.27, 95% CI 0.31–34.72) or pregnancy (OR = 3.46, 95% CI 0.98–12.2) rates. Metformin co-administration in an IVF treatment did not improve the pregnancy rate (OR = 1.29, 95% CI 0.84–1.98) but was associated with a reduction in the risk of ovarian hyperstimulation syndrome (OHSS) (OR = 0.21, 95% CI 0.11–0.41) [65]. However, the authors concluded that the review was inconclusive in terms of not being able to exclude an important clinical treatment effect because of the small number of trials and small sample sizes of the individual trials limiting the power of the meta-analysis.

Neveu et al. [66] carried out an observational comparative study to determine which first-line medication (CC or metformin) was more effective in PCOS patients undergoing OI and to verify whether any patient characteristic was associated with a better response to therapy. The authors included 154 patients who had never been treated for OI to avoid confounding effects of a previous fertility treatment. Patients receiving metformin alone had an increased ovulation rate compared with those receiving CC alone (75.4% vs 50%). Patients on metformin had similar ovulation rates compared with those in the combination group (75.4% vs 63.4%). Pregnancy rates were equivalent in the three groups. Response to metformin was independent of body weight and dose. Finally, non-smoking predicted better ovulatory response overall, as well as lower fasting glucose for CC and lower androgens for metformin.

A literature review [67] was carried out to establish whether metformin was efficacious when given to CC-resistant PCOS patients (the Medline database was searched from January 1, 1980, to January 1, 2005). When the data from four prospective, double-blind, placebo-controlled trials were pooled, the overall effect of the addition of metformin in the CC patient was $p = 0.0006$, with a 95% CI of OR of 1.81–8.84. In only two trials was the randomization prospective; when the data of these two trials were pooled, the overall effect of the addition of metformin in the CC-resistant patient was $p < 0.0001$, with a 95% CI of OR of 6.24–70.27. Combining all data gave an overall positive effect of $p < 0.0001$, with a 95% CI of OR of 3.59–12.96. The authors concluded that the addition of metformin in the CC-resistant patient is highly effective at achieving ovulation. In the largest study

to date, Legro and colleagues [68] randomized 626 sub-fertile women with PCOS who had received previous fertility therapy but were not known to be CC resistant to have CC + placebo, extended-release metformin + placebo, or a combination of metformin + CC for up to six months. The dose of extended-release metformin was gradually increased until a maximum dose of 2000 mg/day. Medication was discontinued when pregnancy was confirmed, and subjects were followed until delivery. The primary endpoint of the study was live birth rate. The live birth rate was 22.5% (47 of 209 subjects) in the CC group, 7.2% (15 of 208) in the metformin group, and 26.8% (56 of 209) in the combination therapy group ($p < 0.001$ for metformin vs both CC and combination therapy; $p = 0.31$ for CC vs combination therapy). Among pregnancies, the rate of multiple pregnancies was 6.0% in the CC group, 0% in the metformin group, and 3.1% in the combination therapy group. The rates of first-trimester pregnancy loss did not differ significantly among the groups. However, the conception rate among subjects who ovulated was significantly lower in the metformin group (21.7%) than in either the CC group (39.5%, $p = 0.002$) or the combination therapy group (46.0%, $p < 0.001$). With the exception of pregnancy complications, adverse event rates were similar in all groups, though gastrointestinal side effects were more frequent and vasomotor and ovulatory symptoms less frequent in the metformin group than in the CC group. The authors concluded that CC was superior to metformin at achieving live birth in women with PCOS, although multiple births are a complication.

In spite of the non-significant difference in live birth rates between CC and combination therapy, the latter group had superior ovulation rates versus CC or metformin alone (60.4% vs 49.0% vs 29.0%; **Figure 39.3**) [68] and a trend to an improvement in the pregnancy rate (absolute difference = 7.2%) following use of CC + metformin versus CC. There were some important reductions in body mass index (BMI), testosterone, insulin, and insulin resistance in patients treated with the combination versus CC alone.

Some of the differences in results reported in Legro et al. [68] compared with Palomba et al. [64] may have been due to the inclusion of a large percentage of patients with a BMI >30 kg/m 2 .

However, in a post hoc analysis, the largest differences in pregnancy rate and live birth rate in the CC versus CC + metformin groups were found in women with a BMI >34 kg/m 2 .

The last Cochrane systematic review by Sharpe et al. [69] examined the use of metformin alone for ovulation induction in women with PCOS. They reported that metformin may be beneficial over placebo for live birth, however, more women probably experienced gastrointestinal side effects. It was uncertain that metformin plus CC improved live birth rates compared to CC alone. From trials where metformin was compared with CC, data for live birth were inconclusive. However, metformin may still be useful as an adjuvant to OI. In a Finnish multicentre study, pregnancy rates increased when metformin was added from three months pre-treatment and in subsequent combination therapy in the obese subgroup with PCOS [70].

In a 2017 Cochrane systematic review, Bordewijk et al. [71] determined the effectiveness of metformin during ovulation induction with gonadotrophins followed by timed intercourse or IUI in PCOS women. There was just a small number of RCTs included (five with 264 women) comparing gonadotrophins plus metformin versus gonadotrophins alone. The authors concluded there was some evidence suggesting metformin addition may increase the live birth rate. At this moment, however, evidence is insufficient to show an effect of metformin on multiple pregnancy rates and adverse events.

Another recent Cochrane systematic review [72] has examined the effectiveness and safety of metformin in women with PCOS, as a co-treatment during IVF or ICSI to achieve pregnancy or live birth. This included 13 RCTs involving a total of 1132 women with PCOS undergoing IVF/ICSI treatments. The authors concluded that in metformin versus placebo/no treatment before or during IVF/ICSI treatment there was no good evidence that metformin improved live birth rates. When a long GnRH-agonist protocol was used, metformin may increase the clinical pregnancy rate but not live birth rate. However, in a routine GnRH-antagonist protocol, metformin may reduce live birth rates. Finally, metformin may reduce the incidence of OHSS but may result in a higher incidence of side effects.

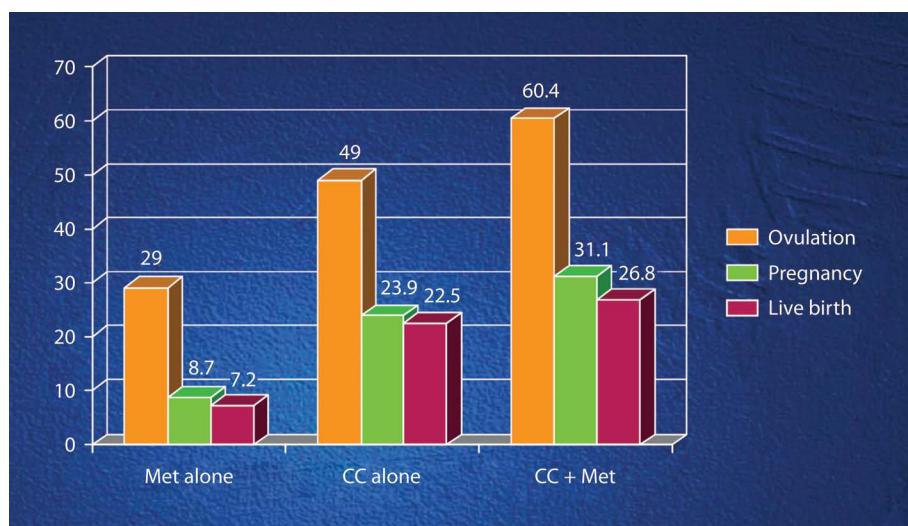


FIGURE 39.3 Ovulation, pregnancy, and live birth rates (%) in polycystic ovary syndrome patients treated with Met alone, CC alone, or Met + CC. Abbreviations: Met, metformin; CC, clomiphene citrate. (Data compiled from fig 2 of reference 68)

To conclude, whereas the adverse features of PCOS can be ameliorated with lifestyle intervention, such as diet and exercise, some further short-term benefits related to ovulation may be derived from medication with metformin. Further studies are warranted to examine the role of metformin in managing the long-term metabolic implications of PCOS.

Pharmacokinetics

Metformin is administered orally and has an absolute bioavailability of 50%–60%, and gastrointestinal absorption is apparently complete within six hours of ingestion. Metformin is rapidly distributed following absorption and does not bind to plasma proteins. No metabolites or conjugates of metformin have been identified. Metformin undergoes renal excretion and has a mean plasma elimination half-life after oral administration of between 4.0 and 8.7 hours. Food decreases the extent of and slightly delays the absorption of metformin.

Side effects and safety

In one US double-blind clinical study of metformin in patients with type 2 diabetes, the most reported adverse reactions (reported in >5% patients) following metformin use were diarrhoea (53%), nausea/vomiting (25.5%), flatulence (12.1%), asthenia (9.2%), indigestion (7.1%), abdominal discomfort (6.4%), and headache (5.7%). Overall, metformin use in women of reproductive age has an assured safety record [70].

Gonadotropins

Human chorionic gonadotropin: The LH surge surrogate

Owing to inconsistency and attenuation of the spontaneous LH surge in COS, and its effective ablation in patients being treated with GnRH agonists, human chorionic gonadotropin (hCG) has been uniformly adopted by all successful ovarian stimulation programs to affect the final triggering of ovulation. When pre-ovulatory follicles are present, administration of hCG is followed by granulosa cell luteinization, a switch from oestradiol to P synthesis, resumption of meiosis and oocyte maturation, and subsequent follicular rupture 36–40 hours later. These processes will occur only if the follicle is of appropriate size and granulosa and theca cell receptivity is adequate, depending on LH receptor status.

Human chorionic gonadotropin has been used as a surrogate LH surge because of the degree of homology between the two hormones. Both LH and hCG are glycoproteins with a molecular weight of approximately 30 kDa, and both have almost identical α -subunits and a high cysteine content (Figure 39.4). Most importantly, they have the same natural function (i.e. to induce luteinization and support lutein cells). Major differences include the sequence of the β -subunit, the regulation of secretion of both hormones, and the pharmacokinetics of clearance of hCG as opposed to LH (Table 39.2) [73, 74].

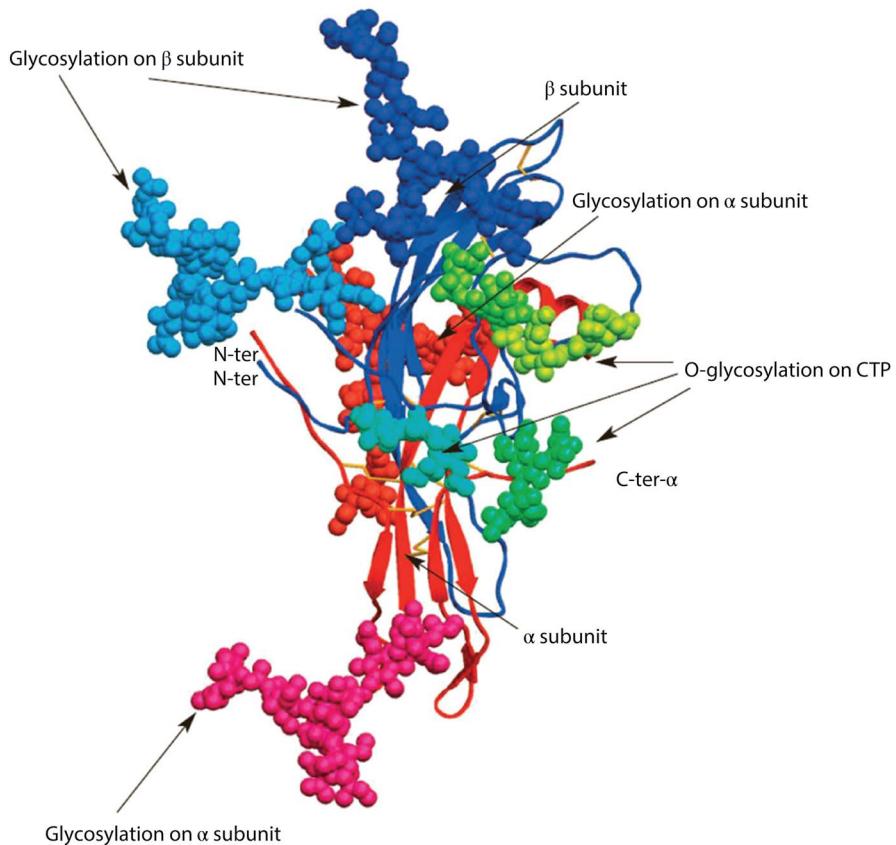


FIGURE 39.4 Human chorionic gonadotropin (hCG) model. Computerized model of hCG with full glycosylation and CTP. Abbreviation: CTP, cytidine triphosphate. (This model was created and provided by the scientific department of Serono Laboratories, United States.)

TABLE 39.2 Luteinizing Hormone (LH) and Human Chorionic Gonadotropin (hCG) Pharmacokinetics and Characteristics. Pharmacokinetics of Recombinant Human LH (rLH), Urinary Human Menopausal Gonadotropin (u-hMG), Urinary hCG (u-hCG), and Recombinant hCG (r-hCG)

Test Drug	rLH	u-hMG	u-hCG	r-hCG
Subjects (n)	12	12	12	12
Route	IV	IV	IV	IV
Dose (IU)	300	300	5000	5000
C_{max}^a (IU/l)	32.1 ± 5.0	24.0 ± 4.2	906 ± 209	1399 ± 317
$t_{1/2}$ (1) ^a (h)	0.8 ± 0.2	0.7 ± 0.2	5.5 ± 1.3	4.7 ± 0.8
$t_{1/2}$ ^a (h)	10.5 ± 7.9	12.4 ± 12.3	31 ± 3	28 ± 3

Source: Modified from le Cotonne JY et al. [74]; Trichard-Lugan et al. [73].

Note: Results are expressed as mean ± SD.

^a Based on serum concentrations measured with immunoradiometric assay (mean ± SD).

Abbreviations: IV, intravenous; C_{max} , maximum concentration; $t_{1/2}$ (1), initial half-life; $t_{1/2}$, terminal half-life.

The plasma metabolic clearance rate of hCG is slower than that of LH (i.e. a rapid disappearance phase in the first five to nine hours after intramuscular [IM] injection and a slower clearance rate in the 1–1.3 days after administration) (Figure 39.5) [74]. The calculated terminal half-life of recombinant hCG is 28–31 hours [73] and for r-LH 10–12 hours [74], as determined after intravenous (IV) administration of the drugs. By day 10 after administration, <10% of the originally administered hCG was measurable [75]. Some authors have advocated the presence

of a serum factor directed against hCG preparations, which significantly prolongs the half-life of hCG administration to women who have received repeated courses of gonadotropins [76]. Others have not found such a correlation [77]. Ludwig et al. suggested that the main differences between LH and hCG lie within the N-linked oligosaccharides and the C-terminal sequence, in which the latter, and especially the O-linked oligosaccharides in this peptide, are responsible for the longer half-life of hCG compared with LH [78].

It is of interest that hCG does not inhibit the subsequent spontaneous LH surge by the intact pituitary, confirming that an ultrashort loop feedback of LH (here hCG) with its own secretion is not functional [79, 80].

It has been found that elevated P levels immediately after hCG administration subsequently induce pituitary LH surges in CC/human menopausal gonadotropin (hMG) cycles [81].

The long serum half-life of hCG is likely to be an undesirable characteristic in clinical practice. Residual hCG may be mistaken for early detection of de novo synthesis of hCG by a newly implanted pregnancy. Additional consequences of hCG administration are the sustained luteotropic effect, development of multiple corpora lutea, and supra-physiologic levels of oestradiol and P synthesis. Sustained high-level stimulation of the corpora lutea may lead to OHSS, a major complication of gonadotropin therapy [82]. Administration of hCG results in an increase in LH-like activity but does not reconstitute the mid-cycle physiologic FSH surge. Another disadvantage of hCG versus the physiologic LH surge is that of higher luteal phase levels of oestradiol and P induced by supra-physiologic hCG concentrations. Excessive levels of circulating oestradiol have been implicated in the relatively high rates of implantation failure and early pregnancy loss observed in ovarian stimulation programs [83, 84]. Another possible disadvantage of the prolonged activity of hCG is that of small-follicle, delayed ovulation, which could be the cause of the development of multiple pregnancies.

Almost universal use of GnRH agonists and pituitary desensitization protocols has made the fear of untimely LH surges relatively obsolete; hence, the timing of the LH-like stimulus with hCG has been given greater flexibility. Tan et al. [85] showed that there was no difference in cycle outcome with random timing of hCG administration over a three-day period. Unfortunately, invalidation of the pituitary mechanism that releases us from an inappropriate LH surge has also made us completely dependent on hCG, with all its inherent problems, for the final stage of ovulation triggering.

Another issue requiring clarification is the minimal effective dose of hCG in order to trigger oocyte maturation and ovulation. In a study examining the minimal effective dose of hCG in IVF [86], dosages of 2000, 5000, and 10,000 IU of urinary hCG (u-hCG) were administered to 88, 110, and 104 women, respectively. No differences in oocyte recovery were noted when comparing the groups that received 5000 and 10,000 IU. However, a significantly lower number of oocytes were aspirated in the 2000-IU group, compared with the 5000- and 10,000-IU groups.

With the development of recombinant technology, r-hCG became available for clinical use, and is as efficacious as u-hCG with the benefit of improved local tolerance [74, 87, 88]. A study in IVF [88] showed that r-hCG 250 µg is at least as effective as 5000 IU of u-hCG. The use of a higher dose of r-hCG, such as 500 µg, resulted in the retrieval of more oocytes, but also a three-fold increase of OHSS. The local reaction at the injection site was significantly better than to the urinary product of equal dose [76].

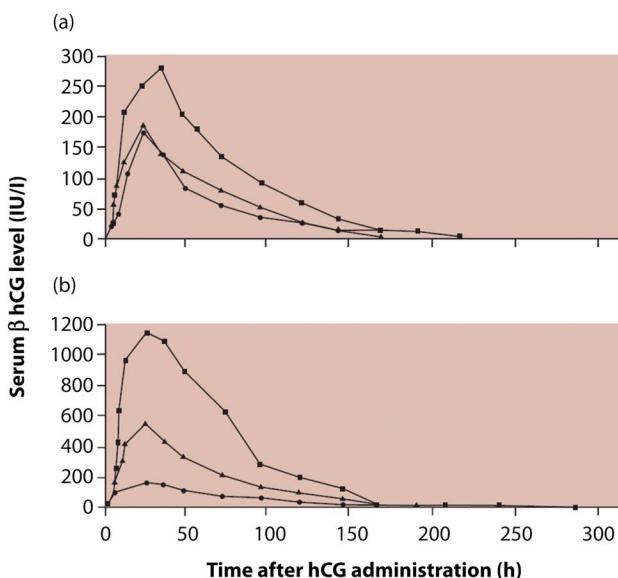


FIGURE 39.5 Pharmacokinetics of serum β -hCG in two hypogonadotropic women: (a) the first woman; (b) the second woman. Three regimens of hCG injections were applied in each woman: 10,000 IU administered subcutaneously or intramuscularly, and 5000 IU administered intramuscularly. Abbreviation: hCG, human chorionic gonadotropin. (Modified from Weissman et al. [75].)

A total of 33 different non-gonadotropin proteins have been recently identified (using classical proteomic analyses) as contaminants in two commercially available preparations of u-hCG [89]. Moreover, human prion peptides were detected in u-hCG (but were not identified in r-hCG) [89].

Gonadotropins: Historical overview

In 1927, Aschheim and Zondek discovered a substance in the urine of pregnant women with the same action as the gonadotropic factor in the anterior pituitary [90]. They called this substance gonadotropin or "prolan". Furthermore, they believed that there were two distinct hormones, prolan A and prolan B. They subsequently used their findings to develop the pregnancy test that carries their names. In 1930, Zondek reported that gonadotropins were also present in the urine of postmenopausal women [91], and in the same year, Cole and Hart found gonadotropins in the serum of pregnant mares [92]. This hormone, pregnant mare serum gonadotropin, was found to have a potent gonadotropic effect in animals. However, it was only in 1937 that Cartland and Nelson were able to produce a purified extract of this hormone [93]. It was not until 1948, because of the work of Stewart, Sano, and Montgomery, that gonadotropins in the urine of pregnant women were shown to originate from the chorionic villi of the placenta, rather than the pituitary. It was subsequently designated "chorionic gonadotropin" [94]. After years of experimental tests, it gradually became apparent that the pituitary factor was needed for the production of mature follicles, and that chorionic gonadotropin could induce ovulation only when mature follicles were present [95]. Within years, it became apparent that the use of gonadotropin extracts from non-primate sources was of limited clinical value owing to the development of antibodies that neutralized their therapeutic effect. In 1947, Piero Donini, a chemist at the Pharmaceutical Institute, Serono, in Rome tried to purify hMG from postmenopausal urine. This purification method was based on a method used by Katzman et al., published in 1943 [96]. The first urine extract of gonadotropin contained LH and FSH and was named Pergonal, inspired by the Italian words "per gonadi" (for the gonads) [97]. The approval to sell Pergonal was first granted by the Italian authorities in 1950 (Table 39.3). Only in 1961, with Pergonal treatment, was the first pregnancy

achieved in a patient with secondary amenorrhoea, which resulted in the birth (in 1962 in Israel) of the first normal baby girl [98]. Urinary FSH (Metrodin) and highly purified FSH became available with the development of new technologies using specific monoclonal antibodies to bind the FSH and LH molecules in the hMG material in such a way that unknown urinary proteins could be removed. Metrodin has a specific activity of 100–200 IU of FSH/mg of protein, whereas Metrodin-HP (highly purified) has an activity of approximately 9000 IU/mg of protein.

Human menopausal gonadotropin

Human menopausal gonadotropin contains an equivalent amount of 75 IU FSH and 75 IU LH *in vivo* bioactivity. Cook et al. [99] demonstrated that hMG preparations also contain up to five different FSH isohormones and up to nine LH species. These differences may cause discrepancies in patients' responses, which are occasionally observed when using various lots of the same preparation.

FSH, which is the major active agent, accounts for <5% of the local protein content in extracted urinary gonadotropin products [100]. The specific activity of these products does not usually exceed 150 IU/mg protein. The different proteins found in various hMG preparations include tumour necrosis factor binding protein I, transferrin, urokinase, Tamm–Horsfall glycoprotein, epidermal growth factor, and immunoglobulin-related proteins [100]. Local side effects, such as pain and allergic reactions, have been reported and attributed to immune reactions related to non-gonadotropin proteins [101].

Technological improvements in recent years have resulted in the introduction of highly purified (HP)-hMG, which can be administered subcutaneously (SC). Highly purified hMG contains more hCG and less LH than does traditional hMG [100]. It was proposed that hMG and HP-hMG induce different follicular development profiles [102]. A total of 33 co-purified proteins were recently identified in HP-hMG products [89]. Importantly, human prion peptides were also detected in hMG and HP-hMG [89, 103]. The identification of human prion proteins in commercially available formulations has prompted careful examination of the risk of transmission of prion disease by urinary gonadotropins [89].

Information is scarce regarding the metabolism of gonadotropin hormones. It was shown that purified preparations of hFSH, hLH, and hCG injected (IV) in humans had serum half-lives (as determined by bioassays) of 180–240 minutes, 38–60 minutes, and 6–8 hours, respectively.

Measuring levels of gonadotropins by *in vivo* bioassays serves to compare biologic effects of gonadotropin preparations in a quantitative manner in animals. In the extensively used Steelman–Pohley assay [104], 21-day-old female Sprague–Dawley rats are injected SC for three days and their ovaries weighed on the fourth day. Disadvantages of this assay are that its sensitivity is too low to detect small amounts of FSH in the serum, reproducibility is poor (+20% variation), and the procedure is cumbersome. The reliance on this assay, in effect, signifies that an ampoule of hMG, which appears to have 75 IU of FSH, may actually contain between 60 and 90 IU. Circulating levels of the gonadotropins measured at any given moment represent the balance between pituitary release and metabolic clearance. After IV injection, the initial half-life of urinary FSH was demonstrated to be approximately two hours [105], and the true terminal (elimination) half-life appeared to be 17 ± 5 hours. After IM injection of urinary FSH preparations, the half-life was estimated to be approximately 35 hours [105].

TABLE 39.3 Milestones of Development in Infertility Treatment

Year	Development
1927	The discovery of pituitary hormone controlling ovarian function
1959	Purification and clinical use of pituitary and urine gonadotropins
1960	Clinical use of clomiphene citrate
1966	Use of clomiphene citrate and gonadotropin becomes common practice
1970	Development of radioimmunoassay for measuring hormone levels
1978	Ultrasound imaging of ovarian follicles
1984	Use of gonadotropin-releasing hormone agonists in infertility treatment
1985	Further purification of urinary gonadotropins
1990	Use of recombinant gonadotropins

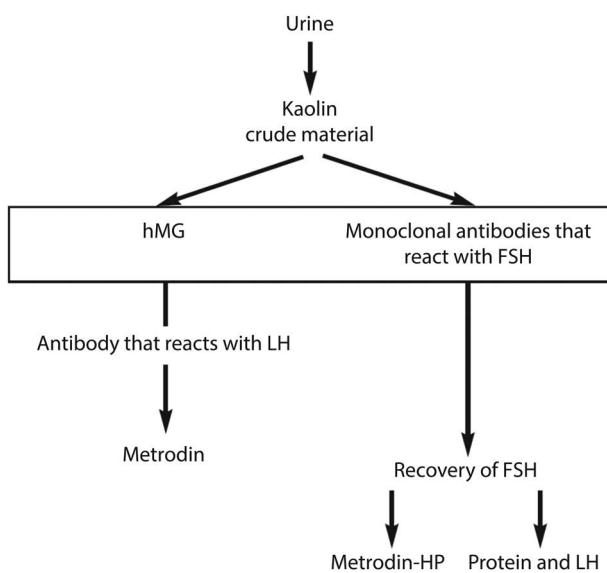


FIGURE 39.6 Schematic presentation of the production of hMG and the purification of urinary FSH and HP-FSH. Abbreviations: FSH, follicle-stimulating hormone; hMG, human menopausal gonadotropin; HP, high-purity; LH, luteinizing hormone.

Purified FSH

Further purification of hMG substantially decreased LH-like activity, leading to a commercial purified FSH (pFSH) preparation. Metrodin was introduced in the mid-1980s and is a product from the same source as hMG, but the LH component has been removed by immunoaffinity chromatography (Figure 39.6).

Apart from obtaining a more purified product, the rationale of developing a pFSH preparation was that OI using gonadotropins in patients with elevated endogenous LH serum levels could, theoretically, preferably be performed without exogenously administered LH. It was also suggested that FSH alone could increase folliculogenesis [106]. Furthermore, it was speculated that LH in gonadotropin preparations could be responsible for the high incidence of complications in patients with elevated serum LH levels [107, 108]. However, other studies [109, 110] have indicated that the effectiveness of gonadotropin preparations and the occurrence of OHSS were not dependent on the LH:FSH ratio.

The desirable goal of having an FSH preparation of high purity led to the development of an immunopurified product (Metrodin-HP) of >95% purity [111].

Recombinant human gonadotropins (FSH, LH, and chorionic gonadotropin)

Following the development of highly purified urinary FSH, considerable improvements have facilitated both separation of FSH from LH and its production using recombinant technology. Early technology focused on the production of biological molecules in bacterial cells (usually *Escherichia coli*). However, the structural complexity of human gonadotropins such as FSH and the need for post-translational modification of the molecule by protein folding and glycosylation made functional protein production impossible in prokaryotes. Thus, a mammalian cell culture system was employed, with functional molecules being produced in Chinese hamster ovary (CHO) cells.

The world's first r-hFSH (follitropin- α) preparation for clinical use was produced by Serono Laboratories in 1988 and was licensed for marketing in the European Union as GONAL-f in 1995. An r-hFSH (follitropin- β ; Puregon) product was also licensed by Organon Laboratories in 1996. The genes for the other gonadotropins have also been transfected into mammalian cell lines, and r-hLH and r-hCG are now commercially available (r-hLH as Luveris, Merck, Germany; r-hCG as Ovidrel/Ovitrelle, Merck; and r-hFSH and r-hLH in a 2:1 ratio, Pergoveris, Merck). However, the following description of manufacturing techniques and physicochemical properties will focus on r-hFSH (follitropin- α).

The production of hFSH by recombinant technology required isolation and cloning of genes for two subunits, the α -subunit—which is also common to hLH and hCG—and a hormone-specific β -subunit. Appropriate vectors were prepared and transfected into suitable immortalized mammalian cell lines. The cell line originally chosen by Serono Laboratories was well established (CHO-DUKX), and already being used to produce proteins such as recombinant human erythropoietin. These cells are normally dihydrofolate reductase deficient, and therefore sensitive to tetrahydrofolate analogues such as methotrexate. Cells were co-transfected with the human α and β FSH genes and then treated with methotrexate, in order to select successfully transfected cells that could express the newly introduced genes.

A stable line of transformed cells was selected, which secreted high quantities of r-hFSH. These cell lines were used to establish a master cell bank (MCB), which now serves as the source of working cell banks (WCBs). The MCB consists of individual vials containing identical cells, which are cryopreserved until required. Thus, a continuous supply of r-hFSH with guaranteed consistency from WCB to WCB is now available by expansion of cells recovered from a single vial of the MCB [112]. MCBs and WCBs are routinely tested for sterility, mycoplasma, and viral contamination.

Quantifying and standardizing gonadotropin content

Traditionally, quantification of hFSH, LH, and hCG for clinical use has involved the use of *in vivo* bioassays. For hFSH, a number of bioassays have been assessed for this purpose, but one of the most robust and specific remains the Steelman-Pohley *in vivo* assay, first developed in the 1950s [104]. FSH activity is quantified by rat ovarian weight gain, and FSH vials or ampoules are subsequently filled according to the desired bioactivity, measured in IU. However, the assay has a number of limitations: it is time-consuming, cumbersome, uses large numbers of rats (which is of ethical concern), and is limited in its precision—the European Pharmacopoeia defines an activity range (80%–125% of the target value) within which an FSH batch is acceptable for clinical use.

Recent advances in the manufacturing process for the r-hFSH follitropin- α , however, enable high batch-to-batch consistency in both isoform profile and glycan species distribution [113, 114]. The most significant advantage of this over other commercially available gonadotropins is that it permits FSH to be quantified reliably by protein content (mass in μg) rather than by biologic activity.

The coefficient of variation for an *in vivo* bioassay is typically $\pm 20\%$, compared with less than 2% for physicochemical analytic techniques, such as size-exclusion HPLC (SE-HPLC) [115, 116]. As a result, Merck quantify their follitropin alfa (GONAL-f), r-hLH, and r-hCG protein by SE-HPLC, a precise and robust assay that results in a significant improvement in batch-to-batch consistency [115].

Physicochemical consistency of r-hFSH: Glycan mapping and isoelectric focusing

Glycan mapping provides a fingerprint of the glycan species of r-hFSH and an estimation of the degree of sialylation of the oligosaccharide chains. For each r-hFSH batch, intact glycan species are released by hydrazinolysis and labelled with a fluorescent derivative. As each glycan molecule is labelled with a single molecule of the dye, the response coefficient is the same for all glycan species, which are separated and detected by anion exchange chromatography and fluorimetry. Results are expressed as the relative percentage of the glycan species grouped as a function of their charge, which is related to the number of sialic acids they carry. The hypothetical charge number, Z , is defined as the sum of the percentage areas under the curve in the neutral, mono-, di-, tri-, and tetra-sialylated glycan regions, multiplied by their corresponding charge [116]. The Z number was demonstrated to be a very precise estimate of the degree of sialylation, with a coefficient of variation of 2% or better.

Evaluation of GONAL-f batch data over time has demonstrated a highly consistent glycoform distribution, which reflects the high consistency of its molecular profile [113, 114, 117]. The second physicochemical technique, isoelectric focusing, is performed in a gel matrix across a pH range of 3.5–7.0. After scanning the gel, the pI values and band intensities of the sample isoforms are compared with the reference standard. The distribution of the main bands from GONAL-f has remained similar to the reference standard over time, indicating a high consistency of isoform distribution [115].

Follitropin- α filled by mass

Between-batch analysis of the ratio of GONAL-f bioactivity, measured in IU using the Steelman–Pohley assay, and protein content, measured in μg by SE-HPLC, has demonstrated a stable, normal distribution of specific activity with no bioreactor run effect [113]. Similarly, drug substance production data over time also confirmed the well-controlled behaviour and consistency of the GONAL-f manufacturing process [113, 114]. The highly consistent physicochemical and biologic properties of the product now permit FSH quantification by SE-HPLC, and vials or ampoules can be filled by mass (FbM) rather than by specific bioactivity. This product is referred to as GONAL-f FbM (Merck).

Once the physicochemical consistency of GONAL-f FbM had been demonstrated, the clinical relevance of the improved manufacturing process was assessed. A total of 131 women were enrolled in a multicentre, double-blind, randomized, parallel-group study comparing the efficacy and safety of four batches each of GONAL-f FbM and GONAL-f filled and released by IU (FbIU) in stimulating multiple follicular development prior to IVF [118]. Adequate levels of ovarian stimulation were achieved with both preparations, resulting in a large number of embryos. The clinical pregnancy rate per treated cycle was 30.3% with the FbM preparation compared with 26.2% with FbIU. Both preparations showed similar levels of adverse events. However, it is the consistency of clinical response between batches that is of particular importance to physicians. The study demonstrated that the improved manufacturing process for the FbM over the FbIU preparation was associated with an improvement in the consistency of ovarian response ($p < 0.039$), including significantly improved between-batch consistency in the clinical pregnancy rate ($p < 0.001$). Compared with GONAL-f FbIU, the FbM preparation reduced the between-batch variability in clinical outcome.

Similar results were also demonstrated in larger studies in ART and OI of GONAL-f FbM versus FbIU [119–122]. In a retrospective study by Balasch et al. [119], the clinical results during the introduction of GONAL-f FbM were compared with standard GONAL-f FbIU. The study included the last 125 patients treated with GONAL-f FbIU and the first 125 patients receiving GONAL-f FbM for ART ovarian stimulation. The patient demographics, oocyte yield, the number of metaphase II oocytes, and the fertilization rates were similar in both groups of patients. However, embryo quality as assessed on day 2 and implantation rates were significantly higher (18.6% vs 28.6%, $p = 0.008$) in the r-hFSH FbM group. Accordingly, in spite of the mean number of embryos transferred being significantly lower in the r-hFSH FbM group, there was a trend for higher clinical pregnancy rates (44% vs 35.2%) in this group of patients. In a large UK multicentre observational study carried out using GONAL-f FbM in 1427 ART patients [120], the safety and efficacy of GONAL-f FbM was confirmed in routine clinical practice. The patients' mean age was 34.3 years and an average of 10.3 oocytes were retrieved. Only 2.7% of the patients who started FSH therapy did not receive hCG. The incidence of severe OHSS was 0.4% and the clinical pregnancy rate per cycle was 29.2%.

In the OI study [122], following use of GONAL-f FbM versus FbIU, fewer patients required an adjustment in the FSH dose (37% vs 60%) and there were fewer cancelled cycles (13% vs 21%) during treatment using a chronic low-dose protocol. Hence, the quality of gonadotropin preparation may play an important role in the consistency of the clinical response, including a reduction in the cycle cancellation [123].

Introduction of biosimilar follitropin- α preparations

Twenty years after the launch of the first r-hFSH preparations (follitropin- α and - β), the field of reproductive medicine is at another very important crossroads, with the introduction of "biosimilar" FSH preparations, which takes innovation at a device level to a new high. For example, Ovaleap (follitropin- α , Theramex UK Ltd) and Bemfola (follitropin- α , Gedeon Richter, Hungary) were granted marketing authorizations by the European Medicines Agency (EMA) in 2013 and 2014 respectively. They are available in a reusable multidose pen device and as single daily dose pens, respectively, and are filled by mass. A biosimilar (i.e. a medicine that has been demonstrated through an exhaustive series of physicochemical *in vitro* and *in vivo* tests) and confirmatory Phase I [124] and Phase III studies [125]) to be similar/equivalent in quality, safety, and efficacy to the reference medicinal product GONAL-f by the EMA. In other words, they bear essentially the same active pharmaceutical ingredient, to be used at the same dose, via the same route, for the same indications as the reference product GONAL-f. It has been postulated—incorrectly—that as a biosimilar FSH has a different FSH isoform profile than the originator FSH, it will have different therapeutic efficacy and safety [126]. Actually, slight variability due to post-translational modifications can occur in any originator product batch [127]. It is therefore expected, based on the reference product batches, that the glycosylation pattern of a biosimilar and reference product will not be identical. A recent feature article by de Mora and Howles (2022) [128] has again reviewed the stringent regulatory pathway for biosimilar registration in EU and the common misconceptions around glycoprotein "sameness." It is important to realize that no glycoprotein product, even an originator (first registered), is identical to itself from batch to batch. What is important for bio-similarity to be claimed is to contain any molecular

differences within the accepted variability range of the originator product. This is exactly what the strict registration requirements of an EU biosimilar does. Another common misconception is to refer to any follow-on product that has been commercialized around the world to be termed a biosimilar. In a meta-analysis by Chua et al. (2021) [129] the authors erroneously describe two of the four products included in the meta-analysis as biosimilars. However, based on the regulatory framework used in the regions where the products have been approved, this may not actually be the case. Many regulatory agencies are not fully compliant with the evidence-based principles followed by the WHO-designated stringent regulatory authorities such as UK MHRA, EMA, FDA, and Canadian, Japanese, and Australian agencies [130]. The important positive impact of biosimilar competition, for instance in Europe, is clear: increased patient access and reduced overall cost of fertility treatment [131].

Unfortunately, this is not a new discussion in reproductive medicine. At the European launch in 1996 of follitropin- α (GONAL-f) and follitropin- β (Puregon/Follistim), efforts were made to differentiate the two products based on "significant differences" in their respective isoform profiles [132]. Both products differed in terms of mammalian cell line employed, the method of gene transfection, purification procedure, and formulation, but eventually numerous comparative studies, registries, and retrospective studies demonstrated that the two products were the same in terms of efficacy (oocytes, embryos, pregnancies, and live births) and safety (incidence of OHSS) [133–135]. Interestingly, based on these differences in structure, between GONAL-f and Puregon/Follistim, the latter would never have been considered comparable and hence registered under the biosimilars regulatory pathway.

Follitropin- δ

The most recent recombinant FSH (FE 999049; Rekovelle, Ferring Switzerland) to enter clinical development has been derived using a cell line of human fetal retinal origin. The amino acid sequences of the α - and β -subunits of FE 999049 are identical to that of natural human FSH, but the sialic acid content of the FSH molecule is higher. Studies in healthy women volunteers comparing the pharmacokinetic and pharmacodynamic properties of FE 999049 to follitropin- α showed that FE 999049 has a longer elimination half-life (30 vs 24 hours) and induces a higher ovarian response when administered at equal doses of biological activity [136]. Based on these differences and a Phase II trial [137], an algorithm was developed for dosing based on anti-Mullerian hormone (AMH) and weight (kg) of the IVF patient.

The results of a Phase III study using this dosing algorithm were published [138]. In this assessor-blind study using a GnRH antagonist protocol, different doses of FE 999049 were administered daily according to an AMH-weight algorithm versus a standard dose of 150 IU per day of follitropin- α in women 18–40 years of age. In the FE 999049 arm the dose was fixed, but with follitropin- α the dose could be increased up to a maximum of 450 IU from day 6 of stimulation. A total of 40% of women recruited in both arms were aged 35 years or older.

In spite of a fixed starting dose of 150 IU follitropin- α in all patients, irrespective of their age (and hence AMH), compared to an individualized approach of FE 999049 dosed according to AMH and weight, the main efficacy and safety results were similar, and there were no significant differences in oocytes retrieved (10.4 ± 6.5 vs 10 ± 5.6), clinical pregnancies (31.6% vs 30.7%), incidence of moderate/severe OHSS (1.4% vs 1.4%), or hospitalization due to OHSS (0.9% vs 0.3%). However, the authors reported under

safety outcomes a significantly higher number of "preventive interventions" for follitropin- α (30 vs 15; $p = 0.005$).

In December 2016, follitropin- δ (Rekovelle) was granted marketing authorization in EU member countries for use in COS for the development of multiple follicles in women undergoing ART use, such as an IVF or ICSI cycle. The registration data was generated using Rekovelle in a GnRH antagonist cycle. It is available as a solution for injection, contained in a cartridge to be used with the Rekovelle injection pen.

It would have been more relevant for current clinical practice if the study design had allowed individualized dosing with follitropin- α , as this would have given a more balanced assessment of the relative merits of follitropin- δ . Another recent trial using the same approach perpetrate the confusing message that follitropin delta reduces safety risks and is more effective regarding gonadotrophin dosage versus conventional dosing [139].

Follitropin- ϵ

Other companies (Glycotope GmbH, Berlin Germany) have also been active in the development of another injectable FSH (FSH-GEX®; follitropin epsilon [- ϵ]), which also has different pharmacodynamic properties from follitropin alfa. Follitropin- ϵ has undergone Phase I and II trials [140, 141]. Following the experience to date with follitropin delta, the question could be raised whether entry of yet another follitropin with different pharmacokinetics compared to tried and tested follitropins as well as urinary FSH will yield any important clinical advantages.

Corifollitropin- α

The range of recombinant gonadotropins available for the treatment of sub-fertility has been expanded through protein engineering. A FSH molecule has been engineered to possess an extended half-life and duration of therapeutic action. This long-acting protein, designated FSH-C-terminal peptide (FSH-CTP, corifollitropin- α), was first described by Bouloux and colleagues in 2001 [142]. FSH-CTP consists of the α -subunit of r-hFSH together with a hybrid β -subunit made up of the β -subunit of hFSH and the C-terminal part of the β -subunit of hCG. FSH-CTP has a longer half-life than standard r-hFSH. FSH-CTP initiates and sustains follicular growth for one week, so one dose can replace the first seven daily injections of gonadotropin in COS. A single dose of FSH-CTP induces multi-follicular growth accompanied by a dose-dependent rise in serum inhibin-B [143]. The first live birth resulting from a stimulation cycle with FSH-CTP was reported in 2003 [144], and further studies have been carried out in sub-fertile patients undergoing ART and OI [145–150]. FSH-CTP was approved in two dose forms in 2010 for use in Europe in ART cycles in combination with a GnRH antagonist.

Two large studies were conducted to demonstrate the non-inferiority of FSH-CTP to r-hFSH (follitropin- β) [148, 149]. A multicentre, randomized, double-blind, double-dummy clinical trial involving 34 centres and 1506 patients weighing 60–90 kg was initially performed (ENGAGE study) [149]. Patients undergoing ART cycles in a standard GnRH antagonist protocol received a single dose of FSH-CTP 150 μ g or daily doses of r-hFSH 200 IU during the first week of stimulation. Ongoing pregnancy rates per cycle initiated were not significantly different for FSH-CTP or r-hFSH (38.9% vs 38.1%, respectively; estimated difference 0.9; $p = 0.71$). The reported incidence of moderate/severe OHSS was 4.1% with corifollitropin- α versus 2.7% with follitropin- β [141].

A further study was conducted to evaluate the efficacy and safety of FSH-CTP in women with low body weight. The ENSURE

study was a multicentre, randomized, double-blind, double-dummy clinical trial involving 19 centres and 396 patients weighing <60 kg undergoing ART [148]. Patients undergoing ART in a standard GnRH antagonist protocol received a single dose of FSH-CTP 100 µg or daily doses of r-hFSH 150 IU during the first week of stimulation. The primary endpoint—the mean (standard deviation [SD]) number of oocytes retrieved per started cycle—was 13.3 (7.3) with FSH-CTP compared with 10.6 (5.9), which was within the predefined equivalence range (-3 to +5 oocytes). The reported incidence of moderate or severe OHSS was 3.4% for corifollitropin-α and 1.6% for follitropin-β [140].

FSH-CTP was developed with the aim of simplifying ART treatment regimens. However, there were concerns regarding the high incidence of OHSS associated with FSH-CTP in published studies and in clinical practice [150, 151]. Investigators of the multicentre, open-label, Phase III TRUST study (designed to assess the immunogenicity of repeated exposure to FSH-CTP) raised concerns regarding the high rate of severe OHSS among their patients [150]. Six of nine patients who received corifollitropin-α at a single centre developed severe OHSS three to five days after hCG administration [150]. Three patients were hospitalized for several days and one experienced a pulmonary embolism despite appropriate therapy [150]. In the TRUST study, 25 patients discontinued treatment after the first or second cycle because of an excessive response to COS or signs or symptoms of OHSS [150]. The overall rate of moderate/severe OHSS in the study was 1.8% in cycle 1, 1.0% in cycle 2, and 0% in cycle 3 [150]. The effects of FSH-CTP cannot be adjusted to individual patient requirements [151]; therefore, careful assessment of patient suitability is required before treatment is commenced.

Because of some of these concerns, the recent focus of research has been in the use of corifollitropin-α in ART patients with a known poor response to FSH [152–158], as well as in hypogonadal men who require long-term FSH therapy if fertility restoration is desired [159].

In one of the aforementioned studies, [157], the authors examined the effect of corifollitropin-α followed by 300 IU daily hMG in a short flare-up GnRH agonist and in a long GnRH agonist protocol in poor responders. They found no significant difference in live birth rates and concluded that both of these protocols are feasible options.

Finally, in a Cochrane systematic review [158], the authors concluded that medium doses (150–180 µg) of long-acting FSH were safe and as effective as daily FSH in women with unexplained subfertility. However, there was evidence of a reduced live birth rate in women receiving lower doses (60–120 µg) compared to daily FSH.

Optimizing outcomes of ovarian stimulation

Safety profile of gonadotropins

Accumulation of data on 1160 babies born after induction of ovulation with gonadotropins [31] revealed that major and minor malformations were found in 63 infants, representing an overall incidence of 54.3/1000 (major malformations 21.6/1000; minor malformations 32.7/1000). This rate of malformation is not significantly different from that of the general population.

Outcomes achieved with r-hFSH versus hMG

r-hFSH and hMG are the gonadotropins that are most frequently used for COS with IVF/ICSI. Outcomes achieved using these gonadotropins have been compared over many years in numerous

retrospective studies, RCTs, and meta-analyses [160–170]. Accumulated data from a Cochrane review suggest that all commercially available gonadotropins have similar efficacy and safety profiles [160]. Indeed, there appears to be little overall difference between r-hFSH and hMG in outcomes of fresh ART cycles.

In 2003, Al-Inany et al. published a meta-analysis that compared r-hFSH with urinary FSH products (hMG, pFSH, and HP-FSH) in IVF/ICSI cycles using a long GnRH agonist protocol [161]. Four of the 20 studies compared hMG with r-hFSH and showed no significant difference between hMG ($n = 603$ cycles) and r-hFSH ($n = 611$ cycles) in terms of clinical pregnancy rate per cycle initiated ($OR = 0.81$, 95% CI 0.63–1.05; $p = 0.11$) [153–156]. A different meta-analysis from 2003 included six RCTs ($n = 2030$) of women undergoing COS for IVF/ICSI [157]. Pooling of data from five RCTs that used a long GnRH agonist protocol showed that hMG resulted in significantly higher clinical pregnancy rates versus r-hFSH ($RR = 1.22$, 95% CI 1.03–1.44). However, there was no difference between groups in ongoing pregnancy rates or live births ($RR = 1.20$, 95% CI 0.99–1.45). A related Cochrane systematic review from 2003 also showed no difference in pooled data from four true RCTs in ongoing pregnancy/live birth rate per woman ($OR = 1.27$, 95% CI 0.98–1.64) [158].

In 2005, Al-Inany et al. published an updated meta-analysis involving eight RCTs and 2031 participants. They showed no significant differences between hMG and r-hFSH in ongoing pregnancy/live birth rate, clinical pregnancy, miscarriage, multiple pregnancy, or moderate/severe OHSS [168]. This group published a third meta-analysis in 2008 including 12 trials involving 1453 hMG cycles and 1484 r-hFSH cycles. They showed a significantly higher live birth rate with hMG versus r-hFSH ($OR = 1.2$, 95% CI 1.01–1.42; $p = 0.04$) and similar rates of OHSS in each group ($OR = 1.21$, 95% CI 0.78–1.86; $p = 0.39$) [160]. Also in 2008, Coomarasamy et al. selected seven RCTs that used a long GnRH agonist protocol [170]. A significant increase in live births per woman randomized was found in favour of hMG versus r-hFSH ($RR = 1.18$, 95% CI 1.02–1.38; $p = 0.03$) [161]. In 2008, Al-Inany et al. published another meta-analysis of six trials involving 2371 participants comparing HP-hMG and r-hFSH in women undergoing IVF/ICSI [169]. No significant difference in the overall ongoing pregnancy/live birth rate was found between the groups. However, when IVF cycles were analysed alone, a significantly higher ongoing pregnancy/live birth rate was found in favour of HP-hMG ($OR = 1.31$, 95% CI 1.02–1.68; $p = 0.03$) [102].

The largest Cochrane meta-analysis of r-hFSH and hMG to date was published in 2011 and included data from 16 RCTs involving 4040 patients undergoing fresh ART cycles [160]. The primary endpoint of this analysis was the number of oocytes retrieved, which was selected in order to estimate directly the gonadotropin effects during COS. The overall conclusion demonstrated that if calculating the fresh transfer cycle only, and not exploring the cumulative live birth rate (LBR), there was no difference in live birth, ovarian hyperstimulation syndrome (OHSS), or clinical pregnancy when r-hFSH was compared to urinary gonadotrophins within any of the downregulation groups. There has recently been an updated Cochrane systematic review and network meta-analysis providing a comprehensive review of ovarian stimulation protocols for assisted reproduction [171]. In summary, they reported an uncertainty of a difference between gonadotrophins in long GnRH agonist protocols for LBR and OHSS; GnRH antagonist with HMG (vs r-hFSH) probably reduces OHSS in high responders and in normal/

high responding patients. Interestingly, LH activity may reduce oocyte number, however the effect on frozen embryo number is as yet uncertain.

A recent study of more than 400,000 IVF cycles has confirmed that the number of oocytes retrieved is a robust surrogate outcome for clinical success [172]. Additionally, a further meta-analysis showed that r-hFSH resulted in the retrieval of significantly more oocytes versus hMG ($p < 0.001$), and a significantly lower dose of r-hFSH versus hMG was required ($p = 0.01$) [173]. No significant difference was observed in baseline adjusted pregnancy rates (RR = 1.04; $p = 0.49$) or in OHSS (RR = 1.47; $p = 0.12$).

Individualization of ovarian stimulation

The objective of fertility treatment is the same for all women—optimization of outcomes with minimization of risks. It has become clear that the “one-size-fits-all” approach to fertility treatment is too simplistic, as each woman’s ovarian response to stimulation is highly variable [174]. Indeed, the use of flexible gonadotropin dosing during ovarian stimulation is now believed to be essential to optimizing cycle outcomes [174].

Accurate prediction of extremes of ovarian response prior to COS would allow tailoring of treatment in the first treatment cycle [175, 176]. Numerous biomarkers predictive of ovarian reserve and response to treatment have been proposed [175–189]. Moreover, various algorithms have been developed in order to calculate the optimum FSH starting dose [176, 178]. The CONSORT treatment algorithm attempted to predict the optimum dose of r-hFSH (follitropin- α) for ART cycles based on individual patient characteristics: age, BMI, basal FSH, and antral follicle count (AFC). This algorithm resulted in an adequate oocyte yield, good pregnancy rate, and low incidence of OHSS. However, cycle cancellation due to an inadequate response occurred frequently in the lowest evaluable dose group (75 IU/day) [176].

Other factors that have been studied as potential predictors of ovarian response to COS include basal FSH, inhibin-B, oestradiol, ovarian volume and vascular flow, and AMH. Numerous studies have demonstrated the value of AMH, a marker of the total developing follicular cohort and the growth of small follicles in the ovary, in predicting ovarian response [179–185]. AMH has been shown to correlate significantly with oocyte yield and live birth rate [181], as well as to predict excessive response to COS [180]. A nomogram for the decline in serum AMH with age has been constructed and will facilitate counselling of patients regarding reproductive potential [184, 185]. Assessment of ovarian reserve by AMH before the first cycle of COS may provide a useful approach to individualizing treatment.

Efforts have also been made to identify markers that accurately predict response to the OI regimen in order to improve the safety, efficiency, and convenience of treatment for women with WHO group II anovulatory infertility [186, 187]. The selection of an appropriate starting dose of r-hFSH would allow physicians to individualize established treatment protocols [186]. This could potentially shorten the time taken to reach the ovulation triggering threshold and reduce the risk of cycle cancellation because of extreme responses to gonadotropins [186]. However, attempts to identify factors predictive of response to OI have had limited success [187, 188]. Several investigators have identified BMI as a marker of response to exogenous FSH and ovulation rates [187, 189]. The importance of BMI as a major determinant of successful ovulation was confirmed in a recent analysis of data from normogonadotropic, oligo-ovulatory, or anovulatory women undergoing an OI using a chronic low-dose, step-up treatment regimen

[186]. In addition, AFC and basal serum FSH concentration were shown to be associated with the response to treatment [186].

An individualized approach to ovarian stimulation is likely to result in optimal treatment outcomes [174]. Determination of the most appropriate single drug or combination of drugs for ovarian stimulation, the daily dose, and the duration of treatment is expected to enhance safety and cost-efficacy [174]. Indeed, the identification of groups of patients who are likely to benefit from each available management strategy is essential [174]. Such an approach would incorporate a wide variety of options based on the anticipated ovarian response.

Adjunctive therapies

Supplementation of FSH with LH, growth hormone, or androgens may also help to improve the ovarian response, and this is discussed in depth in Chapter 55. The use of supplementary LH has attracted the most interest in recent years. The classic “two-cell–two-gonadotropin” model proposed that both FSH and LH are required for oestradiol synthesis. LH binds to theca cells to induce synthesis of androgens, which diffuse out into the circulation and into the granulosa cells where, through the FSH-stimulated action of aromatase, they are converted to oestrogen [190]. Thus, LH regulates and integrates both granulosa and theca cell function during late pre-ovulatory development. At this stage, FSH and LH work together to induce local production of growth factors needed for the paracrine regulation of follicular maturation.

LH supplementation is needed for healthy follicular development and oocyte maturation in patients with HH. In patients with HH, stimulation with FSH alone was significantly less effective than stimulation with FSH plus LH in a study by the European Recombinant Human LH Study Group [191]. Based on these results, a product containing a fixed combination of r-hFSH and r-hLH in a 2:1 ratio (Pergoveris, Merck, registered in EU 2007) was developed for follicular maturation in women with severe gonadotropin deficiency [192].

The clinical utility of the LH ceiling effect was further explored in a series of studies by Loumaye and colleagues. Subsequent findings from a pilot study, demonstrated that high doses of rLH in the late follicular phase suppressed follicular development both in HH and WHO II anovulatory women [193].

Hugues et al. [194] investigated if rLH could be used to achieve mono ovulation for conception *in vivo*. In this elegant placebo-controlled, double-blind study, four doses of rLH (150, 300, 660, 1325 IU) were given daily in the late follicular phase (in combination with a fixed dose of 37.5 IU FSH) to find the optimal dose that could maintain growth of a dominant follicle, whilst leading to atresia of secondary ones. The study was conducted in WHO II anovulatory women who were experiencing an excessive ovarian response to FSH treatment. The results demonstrated that doses up to 660 IU rLH/day increased the proportion of patients developing a single dominant follicle compared to placebo.

The use of GnRH agonists for pituitary downregulation in normogonadotropic women undergoing COS may result in LH levels below those that characterize HH. LH-like activity may be provided using hMG. Studies comparing r-hFSH and hMG have been reported earlier in this chapter and generally show little difference in outcomes. Two meta-analyses of studies comparing outcomes in women receiving supplementary r-hLH with those receiving only r-hFSH also showed no differences between treatment groups [195, 196]. Thus, it is generally accepted that LH supplementation has no benefit in normal responders undergoing COS.

There is, however, some evidence to suggest that LH has benefits in women aged >35 years, and in poor or suboptimal responders to COS [197]. A number of studies have suggested that LH supplementation may improve outcomes in cases of advanced maternal age [198–200]. However, conflicting data have been reported from other studies [201, 202]. LH supplementation may also have benefits for women with a suboptimal response to stimulation, which is characterized by normal follicular development up to cycle days 5–7 followed by a plateau of this response on days 8–10. Suboptimal response may be due to LH-β variant polymorphism [203], or polymorphic variants of the FSH receptor [204, 205]. A significant improvement in fertilization and clinical pregnancy rates has been shown with the addition of r-hLH to r-hFSH in women who required high doses of r-hFSH in previous cycles [206]. A number of other studies had shown evidence of the benefit of LH supplementation in patients with suboptimal response to FSH [207, 208].

However in the ESPART trial [209], the largest RCT study carried out in poor ovarian responder (POR) patients, defined according to the Bologna criteria, no benefit in terms of number of oocytes retrieved or ongoing clinical pregnancy rates were found when LH supplementation (using a FSH:LH, 2:1 ratio product) was compared to FSH alone. Whilst it is clear now that the Bologna criteria encompass a heterogeneous patient population and that applying the POSEIDON criteria may well define better those who will benefit from LH supplementation, a recently published real-world evidence study on more than 9000 low-prognosis patients classified according to the POSEIDON criteria [210] did not demonstrate a benefit of LH supplementation on outcomes. A logistic regression analysis revealed that the POSEIDON grouping, number of embryos obtained, number of ET cycles per patient, number of oocytes collected, female age, duration of infertility, and BMI were relevant predictors for cumulative delivery rate (CDR) ($P < 0.001$). Gonadotrophin type, total gonadotrophin dose, type of GnRH analogue, ovulation trigger were not significantly associated with CDR. To summarize, the use of r-hLH in COS protocols for ART has been reviewed [211] extensively, and, to date, there is still no definitive evidence, from randomized clinical trials, that LH supplementation is beneficial in terms of ongoing pregnancy rates. Recently, there has been presented a comprehensive systematic review and network analysis on ovarian stimulation regimens [171]. Here, as far as LH supplementation was concerned in ART, there was a reduction in the number of oocytes retrieved, thus clearly in line with the early studies in hypogonadotropic and PCO women.

Gonadotropin-releasing hormone

Introduction

Control of gonadotropin secretion is exerted by hypothalamic release of GnRH, initially known as LH-releasing hormone, but the lack of evidence for a specific FSH-releasing hormone prompted a change in terminology. GnRH is produced and released from a group of loosely connected neurons located in the medial basal hypothalamus, primarily within the arcuate nucleus, and in the preoptic area of the ventral hypothalamus. It is synthesized in the cell body, transported along the axons to the synapse, and released in a pulsatile fashion into the complex capillary net of the portal system of the pituitary gland [212].

GnRH was first isolated, characterized, and synthesized independently in 1971 by Andrew Schally and Roger Guillemin, who were subsequently awarded the Nobel Prize for their achievement [213, 214]. GnRH is a decapeptide that, similar to several other

brain peptides, is synthesized as part of a much larger precursor peptide, the GnRH-associated peptide, that has a 56-amino acid sequence. The structure of GnRH is common to all mammals, including humans, and its action is similar in both males and females. GnRH is a single-chain peptide comprising 10 amino acids with crucial functions at positions 1, 2, 3, 6, and 10. Position 6 is involved in enzymatic cleavage, positions 2 and 3 in gonadotropin release, and positions 1, 6, and 10 are important for the three-dimensional structure.

In humans, the critical spectrum of pulsatile release frequencies ranges from the shortest inter-pulse frequency of approximately 71 minutes in the late follicular phase to an interval of 216 minutes in the late luteal phase [215, 216].

GnRH AGONIST

Mechanism of action

Although the exact cellular basis for desensitization of the gonadotroph has not been fully delineated, the extensive use of GnRH agonistic analogues in research facilitated an explosive augmentation of information and knowledge. Acute administration of GnRH agonistic analogues increases gonadotropin secretion (the flare-up effect) and usually requires 7–14 days to achieve a state of pituitary suppression. Prolonged administration of GnRH agonistic analogues leads to downregulation of GnRH receptors. This phenomenon was first shown in 1978, when Knobil and co-workers published their classic paper demonstrating downregulation of gonadotropin secretion by sustained stimulation of the pituitary with GnRH [217]. The agonist-bound receptor is internalized via receptor-mediated endocytosis [218], with kinetics determined by the potency of the analogue. The internalized complex subsequently undergoes dissociation, followed by degradation of the ligand and partial recycling of the receptors [219].

Biosynthesis

Native GnRH is a decapeptide and has a short plasma half-life and is rapidly inactivated by enzymatic cleavage. The initial concept was to create substances that prolong the stimulation of gonadotropin secretion. Analogues with longer half-lives and higher receptor activities were created by a structural change at the position of enzymatic breakdown of GnRH.

The first major step in increasing the potency of GnRH was the substitution of glycine number 10 at the C-terminus. Although 90% of the biological activity is lost with splitting of the 10th glycine, it is predominantly restored with the attachment of NH_2 -ethylamide to the proline at position 9 (220). The second major modification was the replacement of the glycine at position 6 by D-amino acids, which decreases enzymatic degradation. The combination of these two modifications was found to have synergistic biologic activity. Agonistic analogues with D-amino acids at position 6 and NH_2 -ethylamide substituting the Gly10-amide are not only better protected against enzymatic degradation but also exhibit a higher receptor binding affinity. The affinity could be further increased by introduction of larger, hydrophobic, and more lipophilic amino acids at position 6 (Table 39.4). The increased lipophilicity of the agonist is associated with a prolonged half-life, which may be attributed to reduced renal excretion through increased plasma protein binding, or fat tissue storage of non-ionized fat-soluble compounds [220].

Thus, in all analogues, position 6 is substituted with a D-amino acid or a D-amino acid with different radicals. Insertion of D-amino acid blocks degradation and thus leads to more stability

TABLE 39.4 The Structure of Gonadotropin-Releasing Hormone (GnRH) and GnRH Agonistic Analogs

Compound	6th Position										10th Position
Amino acid (no.)	1	2	3	4	5	6	7	8	9	10	
Native GnRH	Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	GlyNH ₂	
Nonapeptides											
Leuprolide						Leu				NHEt	
Buserelin						Ser(O ₂ Bu)				NHEt	
Goserelin						Ser(O ₂ Bu)				AzaGlyNH ₂	
Histrelin						D-His(Bzl)				AzaGlyNH ₂	
Decapeptides											
Nafarelin						2Nal				GlyNH ₂	
Triptorelin						Trp				GlyNH ₂	

and higher receptor affinity (Table 39.4) [221–223]. The agonists leuprolide (D-Leu6, Pr9-NHEt) and buserelin (D-Ser(O₂Bu)6, Pr9-NHEt) contain an ethylamide, and goserelin (D-Ser(O₂Bu)6, Pro9-AzaGlyNH₂) and histrelin (Nt-Bzl-D-His6, Pro9-AzaGlyNH₂) contain azaglycine at position 10 and are, therefore, nonapeptides. Nafarelin (D-Nal(2)6) and triptorelin (D-Trp6) contain the original Gly10-amide and are, therefore, decapeptides.

More than 1000 GnRH analogues have been synthesized and tested, but only a few have been introduced into clinical practice. Differences between analogues are mainly related to methods of administration and potency. The available data usually describe the relative potency of a certain GnRH agonist compared with native GnRH (Table 39.5). It is important to note that this has important implications for IVF practice. The recommended daily dose of GnRHa utilized in an ovarian stimulation protocol was found to be generally higher than the minimum effective dose [224]. Additionally, due to the different amino acid substitutions used by manufacturers at position 6 and 10, the potency of different agonists varies, as well as formulation daily versus depot administration [225]. This complicates the comparison of results obtained across studies using different GnRHa compounds; moreover, these product characteristics certainly impacted the outcome of stimulation when comparing FSH only to FSH/LH (HMG) preparations [226].

All GnRH agonistic analogues are small polypeptide molecules that need to be administered parenterally, as they would otherwise be susceptible to gastrointestinal proteolysis. The oral and rectal administration of analogues is associated with very low biopotency (0.0%–1% vs parenteral administration). Intranasal spray is extremely effective, but the bioavailability is only 3%–5%, and the relatively fast elimination kinetics require frequent dosing (two to six times per day) to obtain continuous stimulation and downregulation [227]. For long-term treatment, a depot formulation is available. The drug is formulated as controlled-release depot preparations with the active substance dissolved, or encapsulated, in biodegradable material. IM injections provide maintained therapeutic levels for 28–35 days. Thus, monthly injections are sufficient for maintaining downregulation.

Side effects

Side effects of GnRH agonist therapy are related to the fall in sex hormone serum concentration. As GnRH agonist interacts with GnRH receptors, which are mainly present in the pituitary, no systemic effects are common. The main symptoms of low serum concentrations of oestrogen are flushes, decreased libido, impotence, vaginal dryness, reduced breast size, and emotional instability. One of the matters of concern is the effect of oestrogen depletion on bone mineral density, as oestrogen is of major

TABLE 39.5 Trade Names, Plasmatic Half-Life, Relative Potency, Route of Administration, and Recommended Dose for the Clinically Available Gonadotropin-Releasing Hormone (GnRH) Analogues

Generic Name	Trade Name	Half-Life	Relative Potency	Administration Route	Recommended Dose
Native GnRH			1	IV, SC	
Nonapeptides					
Leuprolide	Lupron	90 minutes	50–80	SC	500–1000 µg/day
			20–30	IM depot	3.75–7.5 mg/month
Buserelin	Superfact, Supercur	80 minutes	20–40	SC	200–500 µg/day
				Intranasal	300–400 × 3–4/day
Histrelin	Supprelin	<60 minutes	100	SC	100 µg/day
Goserelin	Zoladex	4.5 hours	50–100	SC implant	3.6 mg/month
Decapeptides					
Nafarelin	Synarel	3–4 hours	200	Intranasal	200–400 × 2/day
Triptorelin	Decapeptyl	3–4.2 hours	36–144	SC	100–500 µg/day
				IM depot	3.75 mg/month

Abbreviations: IV, intravenous; SC, subcutaneous.

importance in preventing the development of osteoporosis. A summary of data from different trials [227] showed that GnRH analogue therapy caused significant but reversible bone loss. The mechanism appears to be similar to the development of postmenopausal osteoporosis (i.e. high bone turnover with elevated alkaline phosphatase and osteocalcin levels).

Teratogenic effects

There does not appear to be an increased risk of birth defects or pregnancy wastage in human pregnancies exposed to daily low-dose GnRH agonist therapy in the first weeks of gestation. Although placental transfer of GnRH agonists in pregnant rhesus monkeys was demonstrated, no deleterious effects were observed [228]. From their toxicology studies in animals, no toxic effects were reported by the drug manufacturers [227]. Although several authors claimed a normal outcome of pregnancy following inadvertent administration of a GnRH agonist during early pregnancy [229–232], Ron-El et al. [232] reported the birth of a new-born with a small soft cleft palate. Lahat et al. reported a high incidence of attention-deficit/hyperactivity disorder in a long-term follow-up of children inadvertently exposed to GnRH agonists in early pregnancy [233]. Therefore, as this complication is purely iatrogenic, it should best be avoided.

The use of GnRH agonists for COS in ART cycles is discussed in depth in [Chapter 42](#).

GnRH antagonist

Mechanism of action

Antagonist analogues of GnRH have a direct inhibitory, reversible, suppressive effect on gonadotropin secretion. Antagonistic molecules compete for and occupy pituitary GnRH receptors, thus competitively blocking the access of endogenous GnRH and precluding substantial receptor occupation and stimulation. Suppression attained by GnRH antagonists is immediate (no flare-up effect), and, as receptor loss does not occur, a constant supply of antagonists to the gonadotroph is required to ensure that all GnRH receptors are continuously occupied. Consequently, compared with agonistic analogues, a higher dose range of antagonists is required for effective pituitary suppression ([Table 39.6](#)).

Synthesis of GnRH antagonists

Over the past three decades, thousands of GnRH analogues, both agonists and antagonists, have been synthesized. The first generation of antagonistic analogues were hydrophilic, and contained replacements for His at position 2 and for Trp at position 3. Inhibitory activity increased after incorporation of a D-amino acid at position 6. However, histamine release also increased, resulting in anaphylactic reactions that prevented their clinical

TABLE 39.6 Comparing Mechanisms of Action of Gonadotropin-Releasing Hormone (GnRH) Agonists and Antagonists

GnRH Antagonist	GnRH Agonist
Receptor blockage without receptor activation	Receptor downregulation
Competitive inhibition	Pituitary desensitization
Immediate and dose-dependent suppression	Initial flare-up
Rapid reversibility	Slow reversibility

TABLE 39.7 Structure Formulation of Native Gonadotropin-Releasing Hormone (GnRH) and GnRH Antagonists

Name	Amino Acid Sequence
GnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH ₂
First generation	
4F Ant	NAcΔ1, 1Pro-D4FPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-GlyNH ₂
Second generation	
NalArg	NACD2Nal-D4IFPhe-pTrp-Ser-Tyr-DArg-Leu-Arg-Pro-GlyNH ₂
Detirelix	NACD2Nal-D4CIPhe-pTrp-Ser-Tyr-DHarg(Et2)-Leu-Arg-Pro-DAlaNH ₂
Third generation	
NalGlu	NACD2Nal-D4C7Phe-D3Pal-Ser-Arg-DGlut(AA)-Leu-Arg-Pro-DAlaNH ₂
Antide	NACD2Nal-D4CIPhe-D3Pal-Ser-Lys(Nic)-DDLys(Nic)-Leu-Lys(Isp)Pro-DAlaNH ₂
Org30850	NACD4CIPhe-D4CIPhe-DBal-Ser-Tyr-DLys-Leu-Arg-Pro-DAlaNH ₂
Ramorelix	NACD2Nal-D4CIPhe-DTrp-Ser-Tyr-DSet(Rha)-Leu-Arg-Pro-AzaglyNH ₂
Cetrorelix	NACD2Nal-D4CIPhe-D3Pal-Ser-Tyr-DCit-Leu-Arg-Pro-DAlaNH ₂
Ganirelix	NACD2Nal-D4CIPhe-D3Pal-Ser-Tyr-DHarg(Et2)-Leu-Harg(Et2)-Pro-DAlaNH ₂
A-75998	NACD2Nal-D4CIPhe-D3Pal-Ser-NMeTyr-DLys(Nic)-Leu-Lys(Isp)-Pro-DAlaNH ₂
Azaline B	NACD2Nal-D4CIPhe-D3Pal-Ser-Aph(atz)-DAphe(atz)-Leu-Lys(Isp)-Pro-DAlaNH ₂
Antarelix	NACD2Nal-D4CIPhe-D3Pal-Ser-Tyr-DHcit-Leu-Lys(Isp)-Pro-DAlaNH ₂

use. In third-generation antagonistic analogues, the undesirable risks of anaphylaxis and oedema were eliminated by replacing the D-Arg at position 6 by neutral D-ureidoalkyl amino acids, to produce compounds such as cetrorelix, iturelix, azaline B, ganirelix, abarelix, and antarelix ([Table 39.7](#)) [234–240]. An orally active non-peptide GnRH antagonist (elagolix) was approved by the FDA in 2018 for the treatment of endometriosis-related pain. Others are now either available (relugolix, first registered in Japan for uterine fibroids in 2019; relugolix combined with oestradiol and norethisterone acetate in EU in 2021) or coming into clinical use for the treatment of uterine fibroids (linzagolix in EU 2022). There have been some recent studies published from Japan on the use of relugolix in ovarian stimulation protocols [241, 242].

Safety and tolerability studies

The introduction of GnRH antagonists into clinical use was delayed owing to the property of the first generation of antagonists to induce systemic histamine release and a subsequent general edematogenic state. Studies in rat mast cells confirmed that incorporation of D-Cit at position 6 of antagonists results in reduced histamine release [236, 237]. This characteristic of cetrorelix was first assessed in *in vitro* assays that demonstrated effective plasma concentrations to be significantly lower (<10³) than the median effective dose for systemic histamine secretion, and therefore could confidently be regarded as insignificant. Owing to large disparities in such assays, cetrorelix safety was further tested in *in vivo* settings.

Cetrorelix injected at doses of 1.5 mg/kg SC and 1 and 4 mg/kg IV into rats caused no systemic adverse effects, such as oedema, respiratory dysfunction, or cardiovascular compromise. In these animal studies, no teratogenic effects or detrimental influences on implantation rates or on embryonic development were noted when administered in the periconceptional period. Several thousand human patients have been treated with third-generation GnRH antagonists (i.e. ganirelix, cetrorelix, or abarelix) without evidence of systemic or major local skin reactions, and no cessation of therapy was warranted due to side effects [239, 243–249]. The common side effects observed were injection site reactions and possible nausea, headache, fatigue, and malaise. No drug interactions were demonstrated *in vitro*, with medications metabolized through the cytochrome P450 pathway.

It was suggested that GnRH antagonists may adversely affect oocyte or embryo quality, or the endometrium [250–255]. However, most recent evidence suggests that GnRH antagonists do not diminish oocyte or embryo quality or endometrial receptivity [250–252].

Advantages of GnRH antagonists

The use of GnRH antagonists offers a number of potential advantages over agonists [256]. Prolonged pre-treatment to achieve pituitary downregulation is not required [257]. GnRH antagonists are usually administered only when there is a risk of premature LH surge (usually from days 5–7 of stimulation), so symptoms of hypoestrogenism are rare [258]. Furthermore, lower total doses and fewer days of exogenous gonadotropin stimulation are required versus agonists [259]. Consequently, the total cycle duration is shorter, and subsequent cycles can be initiated rapidly [260, 261].

A meta-analysis including 45 RCTs and 7511 women to compare GnRH antagonist and long GnRH agonist protocols for COS in ART cycles showed no significant differences in the live birth or ongoing pregnancy rates, but a significantly lower incidence of OHSS with GnRH antagonists [262]. Interestingly, the pituitary remains responsive to GnRH stimulation during antagonist co-treatment, so a bolus dose of agonist can be administered (instead of hCG) to trigger final oocyte maturation. This approach may have the potential to reduce further the incidence of OHSS for those at high risk [263, 264], as discussed extensively in Chapter 44.

The reduction in treatment burden (in terms of cycle duration and side effects) and a lower risk of OHSS compared with long agonist protocols means that GnRH antagonists are considered to be “patient-friendly” therapies. GnRH antagonists are being used with increasing frequency in COS protocols, and because of their relative advantages, they have replaced GnRH agonists in many clinics as the protocol of first choice.

The use of GnRH antagonists for COS is ART cycles is discussed in depth in Chapter 43.

References

- Howles CM. Genetic engineering of human FSH (Gonal-F). *Hum Reprod*. 1996;2:172–91.
- Lunenfeld B. Historical perspectives in gonadotrophin therapy. *Hum Reprod Update*. 2004;10:453–67.
- Greenblatt RB, Barfield WE, Jungck EC, Ray AW. Induction of ovulation with MRL/41: Preliminary report. *J Am Med Assoc*. 1961;178:101–4.
- Charles D, Klein T, Lunn SF, Loraine JA. Clinical and endocrinological studies with the isomeric components of clomiphene citrate. *J Obstet Gynaecol Br Commonw*. 1969;76:1100–10.
- Pandya G, Cohen MR. The effect of cis-isomer of clomiphene citrate (cis-clomiphene) on cervical mucus and vaginal cytology. *J Reprod Med*. 1972;8:133–8.
- MacLeod SC, Mitton DM, Parker AS, Tupper WR. Experience with induction of ovulation. *Am J Obstet Gynecol*. 1970;108:814–24.
- Murthy YS, Parakh MC, Arronet GH. Experience with clomiphene and cisclomiphene. *Int J Fertil*. 1971;16:66–74.
- Van Campenhout J, Borreman E, Wyman H, Antaki A. Induction of ovulation with cisclomiphene. *Am J Obstet Gynecol*. 1973;115:321–7.
- Connaughton JF Jr, Garcia CR, Wallach EE. Induction of ovulation with cisclomiphene and a placebo. *Obstet Gynecol*. 1974;43:697–701.
- Mikkelsen TJ, Kroboth PD, Cameron WJ, et al. Single-dose pharmacokinetics of clomiphene citrate in normal volunteers. *Fertil Steril*. 1986;46:392–6.
- Kahwanago I, Heinrichs WL, Herrmann WL. Estradiol “receptors” in hypothalamus and anterior pituitary gland: Inhibition of estradiol binding by SH-group blocking agents and clomiphene citrate. *Endocrinology*. 1970;86:1319–26.
- Etgen AM. Antiestrogens: Effects of tamoxifen, nafoxidine, and CI-628 on sexual behavior, cytoplasmic receptors, and nuclear binding of estrogen. *Horm Behav*. 1979;13:97–112.
- Dickey RP, Holtkamp DE. Development, pharmacology and clinical experience with clomiphene citrate. *Hum Reprod Update*. 1996;2:483–506.
- Kousta E, White DM, Franks S. Modern use of clomiphene citrate in induction of ovulation. *Hum Reprod Update*. 1997;3:359–65.
- Vandenberg G, Yen SS. Effect of anti-estrogenic action of clomiphene during the menstrual cycle: Evidence for a change in the feedback sensitivity. *J Clin Endocrinol Metab*. 1973;37:356–65.
- Clark JH, Markaverich BM. The agonistic–antagonistic properties of clomiphene: A review. *Pharmacol Ther*. 1981;15:467–519.
- Clark JH, Peck EJ Jr, Anderson JN. Oestrogen receptors and antagonism of steroid hormone action. *Nature (London)*. 1974;251:446–8.
- Adashi EY. Clomiphene citrate: Mechanism(s) and site(s) of action—A hypothesis revisited. *Fertil Steril*. 1984;42:331–44.
- Messinis IE, Milungos SD. Future use of clomiphene in ovarian stimulation. Clomiphene in the 21st century. *Hum Reprod*. 1998;13:2362–5.
- Messinis IE, Milungos SD. Current and future status of ovulation induction in polycystic ovary syndrome. *Hum Reprod Update*. 1997;3:235–53.
- Out HJ, Coelingh Bennink HJ. Clomiphene citrate or gonadotrophins for induction of ovulation? *Hum Reprod*. 1998;13:2358–61.
- Hughes E, Collins J, Vandekerckhove P. Clomiphene citrate for unexplained subfertility in women. *Cochrane Database Syst Rev*. 2000;2:CD000057.
- Rossing MA, Daling JR, Weiss NS, et al. Ovarian tumors in a cohort of infertile women. *N Engl J Med*. 1994;331:771–6.
- James WH, Barrett C, Hakim C. Anencephaly, ovulation stimulation, subfertility, and illegitimacy [letter]. *Lancet*. 1973;2:916–17.
- Singh M, Singhi S. Possible relationship between clomiphene and neural tube defects. *J Pediatr*. 1978;93:152.
- Dyson JL, Kohler HG. Anencephaly and ovulation stimulation. *Lancet*. 1973;1:1256–7.
- Sandler B. Anencephaly and ovulation stimulation. *Lancet*. 1973;2:379.
- Field B, Kerr C. Ovulation stimulation and defects of neural-tube closure [letter]. *Lancet*. 1974;2:1511.
- Berman P. Congenital abnormalities associated with maternal clomiphene ingestion [letter]. *Lancet*. 1975;2:878.
- James WH. Anencephaly, ovulation stimulation, and subfertility [letter]. *Lancet*. 1974;1:1353.
- Shoham Z, Zosmer A, Insler V. Early miscarriage and fetal malformations after induction of ovulation (by clomiphene citrate and/or human menotropins), *in vitro* fertilization, and gamete intrafallopian transfer. *Fertil Steril*. 1991;55:1–11.

32. Harlap S. Ovulation induction and congenital malformations. *Lancet*. 1976;2:961.
33. Goss PE, Gwyn KM. Current perspectives on aromatase inhibitors in breast cancer. *J Clin Oncol*. 1994;12:2460–70.
34. Harvey HA. Aromatase inhibitors in clinical practice: Current status and a look to the future. *Semin Oncol*. 1996;23:33–8.
35. Smith IE, Fitzharris BM, McKenna JA, et al. Aminoglutethimide in treatment of metastatic breast carcinoma. *Lancet*. 1978;2:646–9.
36. Lamb HM, Adkins JC. Letrozole: A review of its use in postmenopausal women with advanced breast cancer. *Drugs*. 1998;56: 1125–40.
37. Wiseman LR, Adkins JC. Anastrozole: A review of its use in the management of postmenopausal women with advanced breast cancer. *Drugs Aging*. 1998;13:321–32.
38. Biljan MM, Hcmmings R, Brassard N. The outcome of 150 babies following the treatment with letrozole or letrozole and gonadotrophins. *Fertil Steril*. 2005;84(Suppl):1033.
39. Tulandi T, Martin J, Al-Fadhl R, et al. Congenital malformations among 911 newborns conceived after infertility treatment with letrozole or clomiphene citrate. *Fertil Steril*. 2006;85:1761–5.
40. Badawy A, Shokeir T, Allam AF, et al. Pregnancy outcome after ovulation induction with aromatase inhibitors or clomiphene citrate in unexplained infertility. *Acta Obstet Gynecol Scand*. 2009;88:187–91.
41. Legro RS, Brzyski RG, Diamond MP, et al. Letrozole versus clomiphene for infertility in the polycystic ovary syndrome. *N Engl J Med*. 2014;371:119–29.
42. Diamond MP, Legro RS, Coutifaris C, et al. Letrozole, gonadotropin, or clomiphene for unexplained infertility. *N Engl J Med*. 2015;373:1230–40.
43. Legro RS. Ovulation induction in polycystic ovary syndrome: Current options. *Best Pract Res Clin Obstet Gynaecol*. 2016;37: 152–9.
44. Mitwally MF, Casper RF. Use of an aromatase inhibitor for induction of ovulation in patients with an inadequate response to clomiphene citrate. *Fertil Steril*. 2001;75:305–9.
45. Mitwally MF, Casper RF. Aromatase inhibition improves ovarian response to follicle-stimulating hormone in poor responders. *Fertil Steril*. 2002;77:776–80.
46. Mitwally MF, Casper RF. Aromatase inhibition for ovarian stimulation: Future avenues for infertility management. *Curr Opin Obstet Gynecol*. 2002;14:255–63.
47. Fisher SA, Reid RL, Van Vugt DA, Casper RF. A randomized double-blind comparison of the effects of clomiphene citrate and the aromatase inhibitor letrozole on ovulatory function in normal women. *Fertil Steril*. 2002;78:280–5.
48. Mitwally MF, Casper RF. Aromatase inhibition reduces gonadotrophin dose required for controlled ovarian stimulation in women with unexplained infertility. *Hum Reprod*. 2003;18:1588–97.
49. He D, Jiang F. Meta-analysis of letrozole versus clomiphene citrate in polycystic ovary syndrome. *Reprod Biomed Online*. 2011;23:91–6.
50. Franik S, Eltrop SM, Kremer JAM, Kiesel L, Farquhar C. Aromatase inhibitors (letrozole) for subfertile women with polycystic ovary syndrome. *Cochrane Database Syst Rev*. 2018;(5): CD010287. DOI: [10.1002/14651858.CD010287.pub3](https://doi.org/10.1002/14651858.CD010287.pub3)
51. Tredway D, Schertz JC, Bock D, et al. Anastrozole vs. clomiphene citrate in infertile women with ovulatory dysfunction: A phase II, randomized, dose-finding study. *Fertil Steril*. 2011;95:1720–4.
52. Tredway D, Schertz JC, Bock D, et al. Anastrozole single-dose protocol in women with oligo- or anovulatory infertility: Results of a randomized phase II dose-response study. *Fertil Steril*. 2011;95:1725–9.
53. Griesinger G, von OS, Schultze-Mosgau A, et al. Follicular and endocrine response to anastrozole versus clomiphene citrate administered in follicular phase to normoovulatory women: A randomized comparison. *Fertil Steril*. 2009;91:1831–6.
54. Davar R, Oskouian H, Ahmadi S, Firouzbadi RD. GnRH antagonist/letrozole versus microdose GnRH agonist flare protocol in poor responders undergoing *in vitro* fertilization. *Taiwan J Obstet Gynecol*. 2010;49:297–301.
55. Garcia-Velasco JA, Moreno L, Pacheco A, et al. The aromatase inhibitor letrozole increases the concentration of intraovarian androgens and improves *in vitro* fertilization outcome in low responder patients: A pilot study. *Fertil Steril*. 2005; 84:82–7.
56. Goswami SK, Das T, Chattopadhyay R, et al. A randomized single-blind controlled trial of letrozole as a low-cost IVF protocol in women with poor ovarian response: A preliminary report. *Hum Reprod*. 2004;19:2031–5.
57. Ozmen B, Sonmezler M, Atabekoglu CS, Olmus H. Use of aromatase inhibitors in poor-responder patients receiving GnRH antagonist protocols. *Reprod Biomed Online*. 2009;19:478–85.
58. Verpoest WM, Kolibianakis E, Papanikolaou E, et al. Aromatase inhibitors in ovarian stimulation for IVF/ICSI: A pilot study. *Reprod Biomed Online*. 2006;13:166–72.
59. Bülow NS, Dreyer Holt M, Skouby SO, Birch Petersen K, Englund ALM, Pinborg A, Macklon NS. Co-treatment with letrozole during ovarian stimulation for IVF/ICSI: A systematic review and meta-analysis. *Reprod Biomed Online*. 2022;44(4):717–736. DOI: [10.1016/j.rbmo.2021.12.006](https://doi.org/10.1016/j.rbmo.2021.12.006).
60. Lord JM, Flight IH, Norman RJ. Insulin-sensitising drugs (metformin, troglitazone, rosiglitazone, pioglitazone, d-chiro-inositol) for polycystic ovary syndrome. *Cochrane Database Syst Rev*. 2003;3:CD003053.
61. Lobo RA. Choice of treatment for women with polycystic ovary syndrome. *Fertil Steril*. 2006 Jul;86 Suppl 1:S22–3.
62. UK National Collaborating Centre for Women's and Children's Health. Fertility: Assessment and Treatment for People with Fertility Problems. Clinical Guideline 11. UK: National Institute for Clinical Excellence, 2004. <https://www.nice.org.uk>.
63. Moll E, Bossuyt PM, Korevaar JC, Lambalk CB, van der Veen F. Effect of clomifene citrate plus metformin and clomifene citrate plus placebo on induction of ovulation in women with newly diagnosed polycystic ovary syndrome: Randomised double blind clinical trial. *BMJ*. 2006;332(7556):1485.
64. Palomba S, Orio F Jr., Falbo A, et al. Prospective parallel randomized, double-blind, double-dummy controlled clinical trial comparing clomiphene citrate and metformin as the first-line treatment for ovulation induction in nonobese anovulatory women with polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2005;90: 4068–74.
65. Costello MF, Chapman M, Conway U. A systematic review and meta-analysis of randomized controlled trials on metformin co-administration during gonadotrophin ovulation induction or IVF in women with polycystic ovary syndrome. *Hum Reprod*. 2006;21: 1387–99.
66. Neveu N, Granger L, St-Michel P, Lavoie HB. Comparison of clomiphene citrate, metformin, or the combination of both for first-line ovulation induction and achievement of pregnancy in 154 women with polycystic ovary syndrome. *Fertil Steril*. 2007;87: 113–20.
67. Siebert TI, Kruger TF, Steyn DW, Nosarka S. Is the addition of metformin efficacious in the treatment of clomiphene citrate-resistant patients with polycystic ovary syndrome? A structured literature review. *Fertil Steril*. 2006;86:1432–7.
68. Legro RS, Barnhart HX, Schlaff WD, et al. Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome. *N Engl J Med*. 2007;356:551–66.
69. Sharpe A, Morley LC, Tang T, Norman RJ, Balen AH. Metformin for ovulation induction (excluding gonadotrophins) in women with polycystic ovary syndrome. *Cochrane Database Syst Rev*. 2019;(12):CD013505. DOI: [10.1002/14651858.CD013505](https://doi.org/10.1002/14651858.CD013505).

70. Morin-Papunen L, Rantala AS, Unkila-Kallio L, et al. Metformin improves pregnancy and live-birth rates in women with polycystic ovary syndrome (PCOS): A multicenter, double-blind, placebo-controlled randomized trial. *J Clin Endocrinol Metab.* 2012;97:1492–500.
71. Bordewijk EM, Nahuis M, Costello MF, Van der Veen F, Tso LO, Mol BWJ, van Wely M. Metformin during ovulation induction with gonadotrophins followed by timed intercourse or intrauterine insemination for subfertility associated with polycystic ovary syndrome. *Cochrane Database Syst Rev.* 2017;(1):CD009090. DOI: [10.1002/14651858.CD009090.pub2](https://doi.org/10.1002/14651858.CD009090.pub2).
72. Tso LO, Costello MF, Albuquerque LET, Andriolo RB, Macedo CR. Metformin treatment before and during IVF or ICSI in women with polycystic ovary syndrome. *Cochrane Database Syst Rev.* 2020;(12):CD006105. DOI: [10.1002/14651858.CD006105.pub4](https://doi.org/10.1002/14651858.CD006105.pub4).
73. Trinchard-Lugan I, Khan A, Porchet HC, Munato A. Pharmacokinetics and pharmacodynamics of recombinant human chorionic gonadotropin in healthy male and female volunteers. *Reprod Biomed Online.* 2002;4:106–15.
74. le Cotonne JY, Porchet HC, Beltrami V, Munafò A. Clinical pharmacology of recombinant human luteinizing hormone: Part i. Pharmacokinetics after intravenous administration to healthy female volunteers and comparison with urinary human luteinizing hormone. *Fertil Steril.* 1998;69:189–94.
75. Weissman A, Lurie S, Zalel Y, et al. Human chorionic gonadotropin; Pharmacokinetics of subcutaneous administration. *Gynecol Endocrinol.* 1996;10:273–6.
76. Braunstein GD, Bloch SK, Rasor JL, Winikoff J. Characterization of antihuman chorionic gonadotropin serum antibody appearing after ovulation induction. *J Clin Endocrinol Metab.* 1983;57:1164–72.
77. Diczfalussy E, Harlin J. Clinical-pharmacological studies on human menopausal gonadotropin. *Hum Reprod.* 1988;3:21–7.
78. Ludwig M, Doody KJ, Doody KM. Use of recombinant human chorionic gonadotropin in ovulation induction. *Fertil Steril.* 2003;79:1051–9.
79. Kyle CV, Griffin J, Jarrett A, Odell WD. Inability to demonstrate an ultrashort loop feedback mechanism for luteinizing hormone in humans. *J Clin Endocrinol Metab.* 1989;69:170–6.
80. Nader S, Berkowitz AS. Endogenous luteinizing hormone surges following administration of human chorionic gonadotropin: Further evidence for lack of loop feedback in humans. *J Assist Reprod Genet.* 1992;9:124–7.
81. Demoulin A, Dubois M, Gerdy C, et al. Variations of luteinizing hormone serum concentrations after exogenous human chorionic gonadotropin administration during ovarian hyperstimulation. *Fertil Steril.* 1991;55:797–804.
82. Golan A, Ron-El R, Herman A, et al. Ovarian hyperstimulation syndrome: An update review. *Obstet Gynecol Surv.* 1989;44:430–40.
83. Gidley-Baird AA, O'Neill C, Sinosich MJ, et al. Failure of implantation in human *in vitro* fertilization and embryo transfer patients: The effects of altered progesterone/estrogen ratios in humans and mice. *Fertil Steril.* 1986;45:69–74.
84. Forman R, Fries N, Testart J, et al. Evidence for an adverse effect of elevated serum estradiol concentrations on embryo implantation. *Fertil Steril.* 1988;49:118–22.
85. Tan SL, Balen A, el Hussein E, et al. A prospective randomized study of the optimum timing of human chorionic gonadotropin administration after pituitary desensitization in *in vitro* fertilization. *Fertil Steril.* 1992;57:1259–64.
86. Abdalla HI, Ah-Moye M, Brinsden P, et al. The effect of the dose of human chorionic gonadotropin and the type of gonadotropin stimulation on oocyte recovery rates in an *in vitro* fertilization program. *Fertil Steril.* 1987;48:958–63.
87. Lathi RB, Milki AA. Recombinant gonadotropins. *Curr Womens Health Rep.* 2001;1:157–63.
88. The European Recombinant Human Chorionic Gonadotrophin Study Group. Induction of final follicular maturation and early luteinization in women undergoing ovulation induction for assisted reproduction treatment—Recombinant hCG versus urinary hCG. *Hum Reprod.* 2000;15:1446–51.
89. Van Dorsselaer A, Carapito C, Delalande F, et al. Detection of prion protein in urine-derived injectable fertility products by a targeted proteomic approach. *PLoS ONE.* 2011;6:e17815.
90. Aschheim S, Zondek B. *Klin Wochenschr.* 1928;7:8–9.
91. Zondek B. *Klin Wochenschr.* 1930;9:393–6.
92. Cole H, Hart GH. The potency of blood serum of mares in progressive stages of pregnancy in affecting the sexual maturity of the immature rat. *Am J Physiol.* 1931;93:57–68.
93. Cartland GE, Nelson JW. The preparation and purification of extracts containing the gonad-stimulating hormone of pregnant mare serum. *J Biol Chem.* 1937;19:59–67.
94. Stewart HL, Sano ME, Montgomery TL. *J Clin Endocrinol.* 1948;8:175–88.
95. Kotz HL, Hermann W. A review of the endocrine induction of human ovulation. *Fertil Steril.* 1961;12:375–94.
96. Katzman PA, Godfrid M, Cain CK, Doisy EA. *J Biol Chem.* 1943;148: 501–7.
97. Donini P, Montezemolo R. *Rassegna di Clinica, Terapia e Scienze Affini.* (A Publication of the Biologic Laboratories of the Instituto Serono) 1949;48:3–28.
98. Lunenfeld B, Sulimovici S, Rabau E, Eshkol A. L'Induction de l'ovulation dans les amenorrhées hypophysaires par un traitement de gonado trophines urinaires menopausiques et de gonado trophines chroniques. *C R Soc Française de Gynecol.* 1973;5:1–6.
99. Cook AS, Webster BW, Terranova PF, Keel BA. Variation in the biologic and biochemical characteristics of human menopausal gonadotropin. *Fertil Steril.* 1988;49:704–12.
100. Giudice E, Crisci C, Eshkol A, Papoian R. Composition of commercial gonadotropin preparations extracted from human post-menopausal urine: Characterization of non-gonadotropin proteins. *Hum Reprod.* 1994;9:2291–9.
101. Li TC, Hindle JE. Adverse local reaction to intramuscular injections of urinary-derived gonadotrophins. *Hum Reprod.* 1993;8:1835–6.
102. Al-Inany HG, bou-Setta AM, Aboulghar MA, et al. Highly purified hMG achieves better pregnancy rates in IVF cycles but not ICSI cycles compared with recombinant FSH: A meta-analysis. *Gynecol Endocrinol.* 2009;25:372–8.
103. Kuwabara Y, Mine K, Katayama A, et al. Proteomic analyses of recombinant human follicle-stimulating hormone and urinary-derived gonadotropin preparations. *J Reprod Med.* 2009;54:459–66.
104. Steelman SL, Pohley FM. Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotropin. *Endocrinology.* 1953;53:604–16.
105. le Cotonne JY, Porchet HC, Beltrami V, et al. Clinical pharmacology of recombinant human follicle-stimulating hormone (FSH). I. Comparative pharmacokinetics with urinary human FSH. *Fertil Steril.* 1994;61:669–78.
106. Schoemaker J, Wentz AC, Jones GS, et al. Stimulation of follicular growth with “pure” FSH in patients with anovulation and elevated LH levels. *Obstet Gynecol.* 1978;51:270–7.
107. Raj SG, Berger MJ, Grimes EM, Taymor ML. The use of gonadotropins for the induction of ovulation in women with polycystic ovarian disease. *Fertil Steril.* 1977;28:1280–4.
108. McPaul PB, Traub AI, Thompson W. Treatment of clomiphene citrate-resistant polycystic ovarian syndrome with pure follicle-stimulating hormone or human menopausal gonadotropin. *Fertil Steril.* 1990;53:792–7.
109. Jacobson A, Marshall JR. Ovulatory response rate with human menopausal gonadotropins of varying FSH–LH ratios. *Fertil Steril.* 1969;20:171–5.

110. Louwerens B. The clinical significance of the FSH-LH ratio in gonadotropin preparations of human origin. A review. *Acta Obstet Gynecol Scand.* 1969;48(Suppl 1):31–40.
111. Howles CM, Loumaye E, Giroud D, Luyet G. Multiple follicular development and ovarian steroidogenesis following subcutaneous administration of a highly purified urinary FSH preparation in pituitary desensitized women undergoing IVF: A multicentre European phase III study. *Hum Reprod.* 1994;9: 424–30.
112. Howles CM. Genetic engineering of human FSH (Gonal-F). *Hum Reprod Update.* 1996;2:172–91.
113. le Cotonne JY, Porchet HC, Beltrami V, Howles C. Comparative pharmacokinetics of two urinary human follicle stimulating hormone preparations in healthy female and male volunteers. *Hum Reprod.* 1993;8:1604–11.
114. Driebergen R, Baer G. Quantification of follicle stimulating hormone (follitropin alfa): Is the *in vivo* bioassay still relevant in the recombinant age? *Curr Med Res Opin.* 2003;19:1–6.
115. Bassett RM, Driebergen R. Continued improvements in the quality and consistency of follitropin alfa, recombinant human FSH. *Reprod Biomed Online.* 2005;10:169–77.
116. Hermentin P, Witzel R, Kanzy EJ, et al. The hypothetical n-glycan charge: A number that characterizes protein glycosylation. *Glycobiology.* 1996;6:217–30.
117. Gervais A, Hammel YA, Pelloux S, et al. Glycosylation of human recombinant gonadotropins: Characterization and batch-to-batch consistency. *Glycobiology.* 2003;13:179–89.
118. Hugues JN, Barlow DH, Rosenwaks Z, et al. Improvement in consistency of response to ovarian stimulation with recombinant human follicle stimulating hormone resulting from a new method for calibrating the therapeutic preparation. *Reprod Biomed Online.* 2003;6:185–90.
119. Balasch J, Fabregues F, Penarrubia J, et al. Outcome from consecutive assisted reproduction cycles in patients treated with recombinant follitropin alfa filled-by-bioassay and those treated with recombinant follitropin alfa filled-by-mass. *Reprod Biomed Online.* 2004;8:408–13.
120. Lass A, McVeigh E, UK Gonal-f FbM PMS Group. Routine use of r-hFSH follitropin alfa filled-by mass for follicular development for IVF: A large multicentre observational study in the UK. *Reprod Biomed Online.* 2004;9:604–10.
121. Martinez G, Sanguineti F, Sepulveda J, et al. A comparison between follitropin alpha filled by mass and follitropin alpha filled by bioassay in the same egg donors. *Reprod Biomed Online.* 2007;14: 26–8.
122. Carizza C, Alam V, Yeko T, et al. Gonal-F® filled by mass in ovulation induction. *Hum Reprod Suppl.* 2003;18:O117.
123. Wiklund M, Hugues JN, Howles C. Improving the consistency of ovarian stimulation: Follitropin alfa filled-by-mass. *Reprod Biomed Online.* 2006;12:663–8.
124. Wolzt M, Gouya G, Sator M, Hemetsberger T, Irps C, Rettenbacher M, Vcelar B. Comparison of pharmacokinetic and safety profiles between Bemfola® and Gonal-F® after subcutaneous application. *Eur J Drug Metab Pharmacokinet.* 2016;41:259–65.
125. Rettenbacher M, Andersen AN, Garcia-Velasco JA, et al. A multicentre phase 3 study comparing efficacy and safety of Bemfola® versus Gonal-F® in women undergoing ovarian stimulation for IVF. *Reprod Biomed Online.* 2015;30:504–13.
126. Orvieto R, Seifer DB. Biosimilar FSH preparations: Are they identical twins or just siblings? *Reprod Biol Endocrinol.* Jun 14, 2016;14(1):32.
127. Schiestl M, Stangler T, Torella C, Cepeljnik T, Toll H, Grau R. Acceptable changes in quality attributes of glycosylated biopharmaceuticals. *Nat Biotechnol.* 2011;29:310–2.
128. de Mora F, Howles CM. Overlapping biosimilar and originator follitropin alfa preparations: How much closer can they get? *Drug Discov Today.* 2022;27(8):2071–2075. DOI: [10.1016/j.drudis.2022.04.022](https://doi.org/10.1016/j.drudis.2022.04.022).
129. Chua SJ, Mol BW, Longobardi S, Orvieto R, Venetis CA, Lispi M, Storr A, D'Hooghe T. Biosimilar recombinant follitropin alfa preparations versus the reference product (Gonal-F®) in couples undergoing assisted reproductive technology treatment: A systematic review and meta-analysis. *Reprod Biol Endocrinol.* 2021;19(1):51. DOI: [10.1186/s12958-021-00727-y](https://doi.org/10.1186/s12958-021-00727-y).
130. World Health Organization. List of Stringent Regulatory Authorities (SRAs). <https://www.who.int/initiatives/who-listed-authority-reg-authorities>
131. QuintilesIMS. Impact of Biosimilars Competition in Europe. https://www.medicinesforeurope.com/wp-content/uploads/2017/05/IMS-Biosimilar-2017_V9.
132. Horsman G, Talbot JA, McLoughlin JD, Lambert A, Robertson WR. A biological, immunological and physico-chemical comparison of the current clinical batches of the recombinant FSH preparations Gonal-F and Puregon. *Hum Reprod.* 2000;15:1898–902.
133. Tulppala M, Aho M, Tuuri T, et al. Comparison of two recombinant follicle-stimulating hormone preparations in *in-vitro* fertilization: A randomized clinical study. *Hum Reprod.* 1999;14:2709–15.
134. Harlin J, Csemiczky G, Wramsby H, Fried G. Recombinant follicle stimulating hormone in *in vitro* fertilization treatment-clinical experience with follitropin alpha and follitropin beta. *Hum Reprod.* 2000;15:239–44.
135. Orvieto R, Nahum R, Rabinson J, Ashkenazi J, Anteby EY, Meltcer S. Follitropin-alpha (Gonal-F) versus follitropin-beta (Puregon) in controlled ovarian hyperstimulation for *in vitro* fertilization: Is there any difference? *Fertil Steril.* 2009;91(4 Suppl):1522–5.
136. Olsson H, Sandstrom R, Grundemar L. Different pharmacokinetic and pharmacodynamic properties of recombinant follicle-stimulating hormone (rFSH) derived from a human cell line compared with rFSH from a non-human cell line. *J Clin Pharmacol.* 2014;54:1299–307.
137. Arce JC, Nyboe Andersen A, Fernandez-Sanchez M, et al. Ovarian response to recombinant human follicle- stimulating hormone: A randomized, antimüllerian hormone-stratified, dose-response trial in women undergoing *in vitro* fertilization/intracytoplasmic sperm injection. *Fertil Steril.* 2014;102:1633–40.
138. Nyboe Andersen A, Nelson SM, Fauser BC, García-Velasco JA, Klein BM, Arce JC; ESTHER-1 study group. Individualized versus conventional ovarian stimulation for *in vitro* fertilization: A multicenter, randomized, controlled, assessor-blinded, phase 3 noninferiority trial. *Fertil Steril.* 2017;107(2):387–96.
139. Qiao J, Zhang Y, Liang X, Ho T, Huang HY, Kim SH, Goethberg M, Mannaerts B, Arce JC. A randomised controlled trial to clinically validate follitropin delta in its individualised dosing regimen for ovarian stimulation in Asian IVF/ICSI patients. *Hum Reprod.* 2021 Aug 18;36(9):2452–2462. DOI: [10.1093/humrep/deab155](https://doi.org/10.1093/humrep/deab155).
140. Abd-Elaziz K, Duijkers I, Stöckl L, Dietrich B, Klipping C, Eckert K, Goletz S. A new fully human recombinant FSH (follitropin epsilon): Two phase I randomized placebo and comparator-controlled pharmacokinetic and pharmacodynamic trials. *Hum Reprod.* 2017;7:1–9.
141. Griesinger G, Dietrich B, Stöckl L, Eckert K, Goletz S, Tandler-Schneider A. Fully human glyco-optimized recombinant FSH (follitropin epsilon) - a randomized, comparator-controlled phase II clinical trial. *Reprod Biomed Online.* 2020;40(2):331–341. DOI: [10.1016/j.rbmo.2019.09.003](https://doi.org/10.1016/j.rbmo.2019.09.003).
142. Bouloux PM, Handelsman DJ, Jockenhovel F, et al. First human exposure to FSH-CTP in hypogonadotropic hypogonadal males. *Hum Reprod.* 2001;16:1592–7.
143. Duijkers IJ, Klipping C, Boerrigter PJ, et al. Single dose pharmacokinetics and effects on follicular growth and serum hormones of a long-acting recombinant FSH preparation (FSH-CTP) in healthy pituitary-suppressed females. *Hum Reprod.* 2002;17:1987–93.
144. Beckers NG, Macklon NS, Devroey P, et al. First live birth after ovarian stimulation using a chimeric long-acting human recombinant follicle-stimulating hormone (FSH) agonist (recFSH-CTP) for *in vitro* fertilization. *Fertil Steril.* 2003;79:621–3.

145. Balen AH, Mulders AG, Fauser BC, et al. Pharmacodynamics of a single low dose of longacting recombinant follicle-stimulating hormone (FSH–carboxy terminal peptide, corifollitropin alfa) in women with world health organization group II anovulatory infertility. *J Clin Endocrinol Metab.* 2004;89:6297–304.
146. Devroey P, Fauser BC, Platteeuw P, et al. Induction of multiple follicular development by a single dose of long-acting recombinant follicle-stimulating hormone (FSH–CTP, corifollitropin alfa) for controlled ovarian stimulation before *in vitro* fertilization. *J Clin Endocrinol Metab.* 2004;89:2062–70.
147. Corifollitropin Alfa Dose-finding Study Group. A randomized dose–response trial of a single injection of corifollitropin alfa to sustain multifollicular growth during controlled ovarian stimulation. *Hum Reprod.* 2008;23:2484–92.
148. Corifollitropin Alfa Ensure Study Group. Corifollitropin alfa for ovarian stimulation in IVF: A randomized trial in lower-body-weight women. *Reprod Biomed Online.* 2010;21:66–76.
149. Devroey P, Boostanfar R, Koper NP, et al. A doubleblind, non-inferiority RCT comparing corifollitropin alfa and recombinant FSH during the first seven days of ovarian stimulation using a GnRH antagonist protocol. *Hum Reprod.* 2009;24:3063–72.
150. Norman RJ, Zegers-Hochschild F, Salle BS, et al. Repeated ovarian stimulation with corifollitropin alfa in patients in a GnRH antagonist protocol: No concern for immunogenicity. *Hum Reprod.* 2011;26:2200–8.
151. Santjohanser C. Letter to the editor “Long-acting-FSH (FSH–CTP) in reproductive medicine”. *Gynakologische Endokrinologie.* 2009;3:183.
152. Kolibianakis EM, Venetis CA, Bosdou JK, Zepiridis L, Chatzimeletiou K, Makedos A, Masouridou S, Triantafyllidis S, Mitsoli A, Tarlatzis BC. Corifollitropin alfa compared with follitropin beta in poor responders undergoing ICSI: A randomized controlled trial. *Hum Reprod.* 2015;30:432–40.
153. Polyzos NP, Camus M, Llacer J, Pantos K, Tournaye H. Corifollitropin α followed by menotropin for poor ovarian responders’ trial (COMPORT): A protocol of a multicentre randomised trial. *BMJ Open.* 2013;3:e002938.
154. Polyzos NP, De Vos M, Corona R, Vloeberghs V, Ortega-Hrepich C, Stoop D, Tournaye H. Addition of highly purified HMG after corifollitropin alfa in antagonist-treated poor ovarian responders: A pilot study. *Hum Reprod.* 2013;28:1254–60.
155. Polyzos NP, Devos M, Humaidan P, Stoop D, Ortega-Hrepich C, Devroey P, Tournaye H. Corifollitropin alfa followed by rFSH in a GnRH antagonist protocol for poor ovarian responder patients: An observational pilot study. *Fertil Steril.* 2013;99:422–6.
156. Boostanfar R, Shapiro B, Levy M, Rosenwaks Z, Witjes H, Stegmann BJ, Elbers J, Gordon K, Mannaerts B, Pursue investigators. Large, comparative, randomized double-blind trial confirming noninferiority of pregnancy rates for corifollitropin alfa compared with recombinant follicle-stimulating hormone in a gonadotropin-releasing hormone antagonist controlled ovarian stimulation protocol in older patients undergoing *in vitro* fertilization. *Fertil Steril.* 2015;104:94–103.
157. Polyzos NP, Corona R, Van De Vijver A, Blockeel C, Drakopoulos P, Vloeberghs V, De Vos M, Camus M, Humaidan P, Tournaye H. Corifollitropin alfa followed by hpHMG in GnRH agonist protocols. Two prospective feasibility studies in poor ovarian responders. *Gynecol Endocrinol.* 2015;31:885–90.
158. Pouwer AW, Farquhar C, Kremer JA. Long-acting FSH versus daily FSH for women undergoing assisted reproduction. *Cochrane Database Syst Rev.* 2015;7:CD009577.
159. <https://clinicaltrials.gov/ct2/show/study/NCT01709331?term=corifollitropin&draw=2&rank=2>
160. van Wely M, Kwan I, Burt AL, et al. Recombinant versus urinary gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. *Cochrane Database Syst Rev.* 2011;2:CD005354.
161. Al-Inany H, Aboulghar M, Mansour R, Serour G. Meta-analysis of recombinant versus urinaryderived FSH: An update. *Hum Reprod.* 2003;18:305–13.
162. European and Israeli Study Group on Highly Purified Menotropin versus Recombinant Follicle-Stimulating Hormone. Efficacy and safety of highly purified menotropin versus recombinant follicle-stimulating hormone in *in vitro* fertilization/intracytoplasmic sperm injection cycles: A randomized, comparative trial. *Fertil Steril.* 2002;78:520–8.
163. Gordon UD, Harrison RF, Fawzy M, et al. A randomized prospective assessor-blind evaluation of luteinizing hormone dosage and *in vitro* fertilization outcome. *Fertil Steril.* 2001;75:324–31.
164. Ng EH, Lau EY, Yeung WS, Ho PC. HMG is as good as recombinant human FSH in terms of oocyte and embryo quality: A prospective randomized trial. *Hum Reprod.* 2001;16:319–25.
165. Westergaard LG, Erb K, Laursen SB, et al. Human menopausal gonadotropin versus recombinant follicle-stimulating hormone in normogonadotropic women down-regulated with a gonadotropin-releasing hormone agonist who were undergoing *in vitro* fertilization and intracytoplasmic sperm injection: A prospective randomized study. *Fertil Steril.* 2001;76:543–9.
166. van Wely M, Westergaard LG, Bossuyt PMM, van der Veen F. Effectiveness of human menopausal gonadotropin versus recombinant follicle stimulating hormone for controlled ovarian hyperstimulation in assisted reproductive cycles. A meta-analysis. *Fertil Steril.* 2003;80:1086–93.
167. van Wely M, Westergaard LG, Bossuyt PM, van der Veen F. Human menopausal gonadotropin versus recombinant follicle stimulation hormone for ovarian stimulation in assisted reproductive cycles. *Cochrane Database Syst Rev.* 2003;1:CD003973.
168. Al-Inany H, Aboulghar MA, Mansour RT, Serour GI. Ovulation induction in the new millennium: Recombinant follicle-stimulating hormone versus human menopausal gonadotropin. *Gynecol Endocrinol.* 2005;20:161–9.
169. Al-Inany HG, bou-Setta AM, Aboulghar MA, et al. Efficacy and safety of human menopausal gonadotrophins versus recombinant FSH: A meta-analysis. *Reprod Biomed Online.* 2008;16:81–8.
170. Coomarasamy A, Afnan M, Cheema D, et al. Urinary hMG versus recombinant FSH for controlled ovarian hyperstimulation following an agonist long down-regulation protocol in IVF or ICSI treatment: A systematic review and meta-analysis. *Hum Reprod.* 2008;23:310–5.
171. Melo P, Eapen A, Chung Y, Jeve Y, Price MJ, Sunkara SK, Macklon NS, Bhattacharya S, Khalaf Y, Tobias A, Broekmans F, Khairy M, Gallos I, Coomarasamy A, O-009 Controlled ovarian stimulation (COS) protocols for assisted reproduction: A Cochrane systematic review and network meta-analysis. *Hum Reprod.* 2022 July;37(1):deac104.009. <https://doi.org/10.1093/humrep/deac104.009>
172. Sunkara SK, Rittenberg V, Raine-Fenning N, et al. Association between the number of eggs and live birth in IVF treatment: An analysis of 400 135 treatment cycles. *Hum Reprod.* 2011;26:1768–74.
173. Leheri P, Schertz JC, Ezcurra D. Recombinant human follicle-stimulating hormone produces more oocytes with a lower total dose per cycle in assisted reproductive technologies compared with highly purified human menopausal gonadotrophin: A meta-analysis. *Reprod Biol Endocrinol.* 2010;8:112.
174. Nardo LG, Fleming R, Howles CM, et al. Conventional ovarian stimulation no longer exists: Welcome to the age of individualized ovarian stimulation. *Reprod Biomed Online.* 2011;23:141–8.
175. Olivennes F, Howles CM, Borini A, et al. Individualizing FSH dose for assisted reproduction using a novel algorithm: The CONSORT study. *Reprod Biomed Online.* 2009;18:195–204.
176. Howles CM, Saunders H, Alam V, Engrand P. Predictive factors and a corresponding treatment algorithm for controlled ovarian stimulation in patients treated with recombinant human follicle

- stimulating hormone (follitropin alfa) during assisted reproduction technology (ART) procedures. An analysis of 1378 patients. *Curr Med Res Opin.* 2006;22:907–18.
177. Klinkert ER, Broekmans FJ, Looman CW, et al. The antral follicle count is a better marker than basal follicle-stimulating hormone for the selection of older patients with acceptable pregnancy prospects after *in vitro* fertilization. *Fertil Steril.* 2005;83:811–4.
 178. Popovic-Todorovic B, Loft A, Lindhard A, et al. A prospective study of predictive factors of ovarian response in “standard” IVF/ICSI patients treated with recombinant FSH. A suggestion for a recombinant FSH dosage nomogram. *Hum Reprod.* 2003;18:781–7.
 179. Broer SL, Mol B, Dolleman M, et al. The role of anti-mullerian hormone assessment in assisted reproductive technology outcome. *Curr Opin Obstet Gynecol.* 2010;22:193–201.
 180. Broer SL, Dolleman M, Opmeer BC, et al. AMH and AFC as predictors of excessive response in controlled ovarian hyperstimulation: A meta-analysis. *Hum Reprod Update.* 2011;17:46–54.
 181. Nelson SM, Yates RW, Fleming R. Serum anti-mullerian hormone and FSH: Prediction of live birth and extremes of response in stimulated cycles—Implications for individualization of therapy. *Hum Reprod.* 2007;22:2414–21.
 182. Nelson SM, Yates RW, Lyall H, et al. Anti-mullerian hormone-based approach to controlled ovarian stimulation for assisted conception. *Hum Reprod.* 2009;24:867–75.
 183. Barad DH, Weghofer A, Gleicher N. Utility of age-specific serum anti-mullerian hormone concentrations. *Reprod Biomed Online.* 2011;22:284–91.
 184. Nelson SM, Messow MC, Wallace AM, et al. Nomogram for the decline in serum antimullerian hormone: A population study of 9,601 infertility patients. *Fertil Steril.* 2011;95:736–41.
 185. Nelson SM, Messow MC, McConnachie A, et al. External validation of nomogram for the decline in serum anti-mullerian hormone in women: A population study of 15,834 infertility patients. *Reprod Biomed Online.* 2011;23:204–6.
 186. Howles CM, Alam V, Tredway D, et al. Factors related to successful ovulation induction in patients with WHO group II anovulatory infertility. *Reprod Biomed Online.* 2010;20:182–90.
 187. Imani B, Eijkemans MJ, Faessen GH, et al. Prediction of the individual follicle-stimulating hormone threshold for gonadotropin induction of ovulation in normogonadotropic anovulatory infertility: An approach to increase safety and efficiency. *Fertil Steril.* 2002;77:83–90.
 188. van Wely M, Fauser BC, Laven JS, et al. Validation of a prediction model for the follicle-stimulating hormone response dose in women with polycystic ovary syndrome. *Fertil Steril.* 2006;86:1710–5.
 189. Mulders AG, Laven JS, Eijkemans MJ, et al. Patient predictors for outcome of gonadotrophin ovulation induction in women with normogonadotropic anovulatory infertility: A meta-analysis. *Hum Reprod Update.* 2003;9:429–49.
 190. Kobayashi M, Nakano R, Ooshima A. Immunohistochemical localization of pituitary gonadotrophins and gonadal steroids confirms the “two-cell, two-gonadotrophin” hypothesis of steroidogenesis in the human ovary. *J Endocrinol.* 1990;126:483–8.
 191. European Recombinant Human LH Study Group. Recombinant human luteinizing hormone (LH) to support recombinant human follicle-stimulating hormone (FSH)-induced follicular development in LH and FSH-deficient anovulatory women: A dose-finding study. *J Clin Endocrinol Metab.* 1998;83:1507–14.
 192. Bosch E. Recombinant human FSH and recombinant human LH in a 2:1 ratio combination: A new tool for ovulation induction. *Expert Rev Obstet Gynecol.* 2009;4:491–8.
 193. Loumaye E, Engrand P, Shoham Z, Hillier SG, Baird DT. Clinical evidence for an LH ‘ceiling’ effect induced by administration of recombinant human LH during the late follicular phase of stimulated cycles in world health organization type i and type II anovulation. *Hum Reprod.* 2003;18(2):314–22. DOI: [10.1093/humrep/deg066](https://doi.org/10.1093/humrep/deg066).
 194. Hugues JN, Soussis J, Calderon I, Balasch J, Anderson RA, Romeu A; Recombinant LH Study Group. Does the addition of recombinant LH in WHO group II anovulatory women over-responding to FSH treatment reduce the number of developing follicles? A dose-finding study. *Hum Reprod.* 2005;20(3):629–35. DOI: [10.1093/humrep/deh682](https://doi.org/10.1093/humrep/deh682).
 195. Kolibianakis EM, Kalogeropoulou L, Griesinger G, et al. Among patients treated with FSH and GnRH analogues for *in vitro* fertilization, is the addition of recombinant LH associated with the probability of live birth? A systematic review and meta-analysis. *Hum Reprod Update.* 2007;13:445–52.
 196. Mochtar MH, van der Veen F, Ziech M, van Wely M. Recombinant luteinizing hormone (rLH) for controlled ovarian hyperstimulation in assisted reproductive cycles. *Cochrane Database Syst Rev.* 2007;2:CD005070.
 197. Howles CM. Luteinizing hormone supplementation in ART. In: *How to Improve Your ART Success Rates*. Kovacs G (ed.). London: Cambridge University Press, pp. 99–104, 2011.
 198. Bosch E, Labarta E, Simón C, et al. The impact of luteinizing hormone supplementation in gonadotropin releasing hormone antagonist cycles. An age adjusted randomized trial. *Fertil Steril.* 2008;90:S41.
 199. Humaidan P, Bungum M, Bungum L, Yding AC. Effects of recombinant LH supplementation in women undergoing assisted reproduction with GnRH agonist down-regulation and stimulation with recombinant FSH: An opening study. *Reprod Biomed Online.* 2004;8:635–43.
 200. Marrs R, Meldrum D, Muasher S, et al. Randomized trial to compare the effect of recombinant human FSH (follitropin alfa) with or without recombinant human LH in women undergoing assisted reproduction treatment. *Reprod Biomed Online.* 2004;8:175–82.
 201. Matorras R, Prieto B, Exposito A, et al. Mid-follicular LH supplementation in women aged 35–39 years undergoing ICSI cycles: A randomized controlled study. *Reprod Biomed Online.* 2009;19:879–87.
 202. Fabregues F, Creus M, Penarrubia J, et al. Effects of recombinant human luteinizing hormone supplementation on ovarian stimulation and the implantation rate in down-regulated women of advanced reproductive age. *Fertil Steril.* 2006;85:925–31.
 203. Nilsson C, Pettersson K, Millar RP, et al. Worldwide frequency of a common genetic variant of luteinizing hormone: An international collaborative research. International Collaborative Research Group. *Fertil Steril.* 1997;67:998–1004.
 204. de Castro F, Moron FJ, Montoro L, et al. Human controlled ovarian hyperstimulation outcome is a polygenic trait. *Pharmacogenetics.* 2004;14:285–93.
 205. Simoni M, Nieschlag E, Gromoll J. Isoforms and single nucleotide polymorphisms of the FSH receptor gene: Implications for human reproduction. *Hum Reprod Update.* 2002;8:413–21.
 206. Lisi F, Rinaldi L, Fishel S, et al. Use of recombinant FSH and recombinant LH in multiple follicular stimulation for IVF: A preliminary study. *Reprod Biomed Online.* 2001;3:190–4.
 207. De Placido G, Alviggi C, Mollo A, et al. Effects of recombinant LH (rLH) supplementation during controlled ovarian hyperstimulation (COH) in normogonadotropic women with an initial inadequate response to recombinant FSH (rFSH) after pituitary downregulation. *Clin Endocrinol.* 2004;60:637–43.
 208. Ferraretti AP, Giaranoli L, Magli MC, et al. Exogenous luteinizing hormone in controlled ovarian hyperstimulation for assisted reproduction techniques. *Fertil Steril.* 2004;82:1521–6.
 209. Humaidan P, Chin W, Rogoff D, D’Hooghe T, Longobardi S, Hubbard J, Schertz J; ESPART Study Investigators. Efficacy and safety of follitropin alfa/lutropin alfa in ART: A randomized controlled trial in poor ovarian responders. *Hum Reprod.* 2017;32(3):544–55. DOI: [10.1093/humrep/dew360](https://doi.org/10.1093/humrep/dew360). Erratum in: *Hum Reprod.* 2017 Jul 1;32(7):1537–8.

210. Esteves SC, Yarali H, Vuong LN, Carvalho JF, Özbeş İY, Polat M, Le HL, Pham TD, Ho TM, Humaidan P, Alvgi C. Cumulative delivery rate per aspiration IVF/ICSI cycle in POSEIDON patients: A real-world evidence study of 9073 patients. *Hum Reprod.* 2021 Jul 19;36(8):2157–2169. DOI: [10.1093/humrep/deab152](https://doi.org/10.1093/humrep/deab152).
211. Mochtar MH, Danhof NA, Ayeleke RO, Van der Veen F, van Wely M. Recombinant luteinizing hormone (rLH) and recombinant follicle stimulating hormone (rFSH) for ovarian stimulation in IVF/ICSI cycles. *Cochrane Database Syst Rev.* 2017;(5):CD005070. DOI: [10.1002/14651858.CD005070.pub3](https://doi.org/10.1002/14651858.CD005070.pub3).
212. Carmel PW, Araki S, Ferin M. Pituitary stalk portal blood collection in rhesus monkeys: Evidence for pulsatile release of gonadotropin-releasing hormone (GnRH). *Endocrinology.* 1976;99:243–8.
213. Schally AV, Arimura A, Kastin AJ, et al. Gonadotropin-releasing hormone: One polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. *Science.* 1971;173:1036–8.
214. Amoss M, Burgus R, Blackwell R, et al. Purification, amino acid composition and n-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. *Biochem Biophys Res Commun.* 1971;44:205–10.
215. Backstrom CT, McNeilly AS, Leask RM, Baird DT. Pulsatile secretion of LH, FSH, prolactin, oestradiol and progesterone during the human menstrual cycle. *Clin Endocrinol.* 1982;71:29–42.
216. Reame N, Sauder SE, Kelch RP, Marshall JC. Pulsatile gonadotropin secretion during the human menstrual cycle: Evidence for altered frequency of gonadotropin-releasing hormone secretion. *J Clin Endocrinol Metab.* 1984;59:328–37.
217. Belchetz PE, Plant TM, Nakai Y, et al. Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone. *Science.* 1978;202:631–3.
218. Suarez-Quian CA, Wynn PC, Catt KJ. Receptor mediated endocytosis of GnRH analogs: Differential processing of gold-labeled agonist and antagonist derivatives. *J Steroid Biochem.* 1986;24:183–92.
219. Schwartz I, Hazum E. Internalization and recycling of receptor-bound gonadotropin-releasing hormone agonist in pituitary gonadotropes. *J Biol Chem.* 1987;262:17046–50.
220. Karten MJ, Rivier JE. Gonadotropin-releasing hormone analog design. Structure–function studies toward the development of agonists and antagonists: Rationale and perspective. *Endocrinol Rev.* 1986;7:44–66.
221. Coy DH, Labrie F, Savary M, et al. LH-releasing activity of potent LH-RH analogs *in vitro*. *Biochem Biophys Res Commun.* 1975;67:576–82.
222. Lemay A, Metha AE, Tolis G, et al. Gonadotropins and estradiol responses to single intranasal or subcutaneous administration of a luteinizing hormone releasing hormone agonist in the early follicular phase. *Fertil Steril.* 1983;39:668–73.
223. Fogelman I. Gonadotropin-releasing hormone agonists and the skeleton. *Fertil Steril.* 1992;57:715–24.
224. Janssens RM, Vermeiden JP, Lambalk CB, Schats R, Schoemaker J. Gonadotrophin-releasing hormone agonist dose-dependency of pituitary desensitization during controlled ovarian hyperstimulation in IVF. *Hum Reprod.* 1998;13(9):2386–91. DOI: [10.1093/humrep/13.9.2386](https://doi.org/10.1093/humrep/13.9.2386).
225. Loumaye E, Naor Z, Catt KJ. Binding affinity and biological activity of gonadotropin releasing hormone agonists in isolated pituitary cells. *Endocrinology.* 1982;111(3):730–6. DOI: [10.1210/endo-111-3-730](https://doi.org/10.1210/endo-111-3-730).
226. Smitz J, Andersen AN, Devroey P, Arce JC, MERIT Group. Endocrine profile in serum and follicular fluid differs after ovarian stimulation with hMG or recombinant FSH in IVF patients. *Hum Reprod.* 2007;22(3):676–87. DOI: [10.1093/humrep/del445](https://doi.org/10.1093/humrep/del445).
227. Brogden RN, Buckley MM, Ward A. Buserelin. A review of its pharmacodynamic and pharmacokinetic properties, and clinical profile. *Drugs.* 1990;39:399–437.
228. Sopelak VM, Hodgen GD. Infusion of gonadotropin releasing hormone agonist during pregnancy: Maternal and fetal responses in primates. *Am J Obstet Gynecol.* 1987;156:755–60.
229. Golan A, Ron-El R, Herman A, et al. Fetal outcome following inadvertent administration of long-acting DTRP6 GnRH micro-capsules during pregnancy: A case report. *Hum Reprod.* 1990;5:123–4.
230. Dicker D, Goldman JA, Vagman I, et al. Pregnancy outcome following early exposure to maternal luteinizing-hormone-releasing hormone agonist (buserelin). *Hum Reprod.* 1989;4:250–1.
231. Weissman A, Shoham Z. Favourable pregnancy outcome after administration of a long-acting gonadotrophin-releasing hormone agonist in the mid-luteal phase. *Hum Reprod.* 1993;8:496–7.
232. Ron-El R, Golan A, Herman A, et al. Midluteal gonadotropin-releasing hormone analog administration in early pregnancy. *Fertil Steril.* 1990;53:572–4.
233. Lahat E, Raziel A, Friedler S, et al. Long-term follow-up of children born after inadvertent administration of a gonadotrophin-releasing hormone agonist in early pregnancy. *Hum Reprod.* 1999;14:2656–60.
234. Bajusz S, Kovacs M, Gazdag M, et al. Highly potent antagonists of luteinizing hormone-releasing hormone free of edematogenic effects. *Proc Natl Acad Sci USA.* 1988;85:1637–41.
235. Ljungqvist A, Feng DM, Hook W, et al. Antide and related antagonists of luteinizing hormone release with long action and oral activity. *Proc Natl Acad Sci USA.* 1988;85:8236–40.
236. Rivier J, Porter J, Hoeger C, et al. Gonadotropin-releasing hormone antagonists with n omega-triazolylornithine, -lysine, or -p-aminophenylalanine residues at positions 5 and 6. *J Med Chem.* 1992;35:4270–8.
237. Nestor JJ Jr, Tahilramani R, Ho TL, et al. Potent gonadotropin releasing hormone antagonists with low histamine-releasing activity. *J Med Chem.* 1992;35:3942–8.
238. Garnick MB, Campion M. Abarelix Depot, a GnRH antagonist, v. LHRH superagonists in prostate cancer: Differential effects on follicle-stimulating hormone. *Abarelix Depot Study Group. Mol Urol.* 2000;4:275–7.
239. Cook T, Sheridan WP. Development of GnRH antagonists for prostate cancer: New approaches to treatment. *Oncologist.* 2000;5:162–8.
240. Deghenghi R, Boutignon F, Wuthrich P, Lenaerts V. Antarelix (EP 24332) a novel water soluble LHRH antagonist. *Biomed Pharmacother.* 1993;47:107–10.
241. Hamada M, Horikawa M, Ensaka C, Enomoto M, Ishii R, Toriumi R, Tachibana N, Taketani Y. A novel orally active gonadotropin-releasing hormone antagonist, relugolix, is a potential substitute for injectable GnRH antagonists in controlled ovarian stimulation in assisted reproductive technology. *Reprod Med Biol.* 2021 Nov 12;21(1):e12424. DOI: [10.1002/rmb2.12424](https://doi.org/10.1002/rmb2.12424).
242. Komiya S, Tsuzuki-Nakao T, Asai Y, Inoue T, Morimoto Y, Okada H. The novel oral gonadotropin-releasing hormone receptor antagonist relugolix is a new option for controlled ovarian stimulation cycles. *Reprod Med Biol.* 2022 Feb 17;21(1):e12448. DOI: [10.1002/rmb2.12448](https://doi.org/10.1002/rmb2.12448).
243. Felberbaum R, Diedrich K. Ovarian stimulation for *in vitro* fertilization/intracytoplasmic sperm injection with gonadotrophins and gonadotrophin releasing hormone analogues: Agonists and antagonists. *Hum Reprod.* 1999;14(Suppl 1):207–21.
244. Bajusz S, Csernus VJ, Janaky T, et al. New antagonists of LHRH. II. Inhibition and potentiation of LHRH by closely related analogues. *Int J Pept Protein Res.* 1988;32:425–35.
245. Diedrich K, Diedrich C, Santos E, et al. Suppression of the endogenous luteinizing hormone surge by the gonadotrophin-releasing hormone antagonist cetrorelix during ovarian stimulation. *Hum Reprod.* 1994;9:788–91.
246. Felberbaum RE, Albano C, Ludwig M, et al. Ovarian stimulation for assisted reproduction with hMG and concomitant midcycle administration of the GnRH antagonist cetrorelix according to the multiple dose protocol: A prospective uncontrolled phase III study. *Hum Reprod.* 2000;15:1015–20.

247. Borm G, Mannaerts B. Treatment with the gonadotrophin-releasing hormone antagonist ganirelix in women undergoing ovarian stimulation with recombinant follicle stimulating hormone is effective, safe and convenient: Results of a controlled, randomized, multicentre trial. The European Orgalutran Study Group. *Hum Reprod.* 2000;15:1490–8.
248. The European Middle East Orgalutran Study Group. Comparable clinical outcome using the GnRH antagonist ganirelix or a long protocol of the GnRH agonist triptorelin for the prevention of premature LH surges in women undergoing ovarian stimulation. *Hum Reprod.* 2001;16:644–51.
249. Fluker M, Grifo J, Leader A, et al. Efficacy and safety of ganirelix acetate versus leuprolide acetate in women undergoing controlled ovarian hyperstimulation. *Fertil Steril.* 2001;75:38–45.
250. Mannaerts B, Gordon K. Embryo implantation and GnRH antagonists: GnRH antagonists do not activate the GnRH receptor. *Hum Reprod.* 2000;15:1882–3.
251. Demirel LC, Weiss JM, Polack S, et al. Effect of the gonadotropin-releasing hormone antagonist ganirelix on cyclic adenosine monophosphate accumulation of human granulosa-lutein cells. *Fertil Steril.* 2000;74:1001–7.
252. Ortmann O, Weiss JM, Diedrich K. Embryo implantation and GnRH antagonists: Ovarian actions of GnRH antagonists. *Hum Reprod.* 2001;16:608–11.
253. Raga F, Casan EM, Kruessel J, et al. The role of gonadotropin-releasing hormone in murine preimplantation embryonic development. *Endocrinology.* 1999;140:3705–12.
254. The Ganirelix Dose-finding Study Group. A doubleblind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist ganirelix (org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon). *Hum Reprod.* 1998;13:3023–31.
255. Rackow BW, Kliman HJ, Taylor HS. GnRH antagonists may affect endometrial receptivity. *Fertil Steril.* 2008;89:1234–9.
256. Prapas N, Prapas Y, Panagiotidis Y, et al. GnRH agonist versus GnRH antagonist in oocyte donation cycles: A prospective randomized study. *Hum Reprod.* 2005;20:1516–20.
257. Saucedo de la Llata E, Moraga Sanchez MR, Batiza RV, et al. Comparison of GnRH agonists and antagonists in an ovular donation program. *Ginecol Obstet Mex.* 2004;72:53–6.
258. Vlahos NF, Bankowski BJ, Zucar HA, et al. An oocyte donation protocol using the GnRH antagonist ganirelix acetate, does not compromise embryo quality and is associated with high pregnancy rates. *Arch Gynecol Obstet.* 2005;272:1–6.
259. Devroey P, Aboulghar M, Garcia-Velasco J, et al. Improving the patient's experience of IVF/ICSI: A proposal for an ovarian stimulation protocol with GnRH antagonist co-treatment. *Hum Reprod.* 2009;24:764–74.
260. Reissmann T, Felberbaum R, Diedrich K, et al. Development and applications of luteinizing hormone-releasing hormone antagonists in the treatment of infertility: An overview. *Hum Reprod.* 1995;10:1974–81.
261. Tur-Kaspa I, Ezcurra D. GnRH antagonist, cetrorelix, for pituitary suppression in modern, patient-friendly assisted reproductive technology. *Expert Opin Drug Metab Toxicol.* 2009;5:1323–36.
262. Al-Inany HG, Youssef MA, Aboulghar M, et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev.* 2011;5:CD001750.
263. Youssef MA, Van d V, Al-Inany HG, et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist assisted reproductive technology cycles. *Cochrane Database Syst Rev.* 2011;1:CD008046.
264. Griesinger G, Schultz L, Bauer T, et al. Ovarian hyperstimulation syndrome prevention by gonadotropin-releasing hormone agonist triggering of final oocyte maturation in a gonadotropin-releasing hormone antagonist protocol in combination with a “freeze-all” strategy: A prospective multicentric study. *Fertil Steril.* 2011;95:2029–33, 2033.e1.

THE ROLE OF FOLLICLE-STIMULATING HORMONE AND LUTEINIZING HORMONE IN OVARIAN STIMULATION

Current Concepts

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Introduction

Current practice of ovarian stimulation (OS) for *in vitro* fertilization (IVF) has the possibility of using different protocols depending on the choice the pituitary suppression regimen and the diverse gonadotropin preparations [1]. With regard to the latter, a major debate continues regarding using pure follicle-stimulating hormone (FSH)-alone regimens or administering some kind of luteinizing hormone (LH) activity-containing preparations.

Although the physiological role of LH during the follicular phase of a natural cycle is unquestionable [2, 3], its impact during an OS cycle outcome and the need for adding it as a supplement remain controversial. A number of studies have analysed this topic, but the conclusions are still confusing; although there is evidence supporting that there is no benefit of LH activity supplementation in an unselected population [4], it is also stated that it might be useful in some particular situations of FSH and LH deficiency, especially in poor responders and older patients [5].

The present chapter is a mini-review on the role of LH activity administration in OS for IVF/intracytoplasmic sperm injection, in which the ultimate action of LH when administered and its impact on ovarian response and cycle outcome are analysed.

Basic physiological background

The initial steps of follicular maturation are independent of gonadotropin action [6]. However, from the early antral follicular stage, follicles become sensitive to the action of gonadotropins. FSH is required to start the development from antral follicles, and this first period is FSH dependent, while LH promotes androgen secretion by theca cells and is implicated in processes related to ovulation itself: follicular dominance, complete maturation (which depends on the follicle transfer of FSH dependency to LH dependency) [7], ovulation, and support of the corpus luteum [8].

The amount of LH necessary to induce a response in the follicle varies from a minimum (“LH threshold”) to a maximum (“LH ceiling”) [9]. This amount has not been determined, but it has been suggested that less than 1% of follicular LH receptors need to be occupied in order to produce a steroidogenic response [10].

Of around 1000 recruited follicles per cycle, only one will be dominant and the others will suffer atresia [11]. The presence of FSH and LH is vital in this complex process.

LH receptors are located in the membranes of theca cells, granulosa cells, interstitial cells, and luteal cells, but also in cells of different tissues, including the endometrium, cervix, and tubal epithelium [12]. These receptors have high affinity and selectivity to bind their respective glycoproteins, and their expression is induced by FSH [13].

Levels of LH vary during the cycle in response to pulsatile liberation of gonadotropin-releasing hormone (GnRH). Acid forms of the gonadotropin are more common in the follicular phase, whereas the alkaline forms are more common in the luteal phase [14]. In the absence of LH, ovarian follicle growth is arrested when the FSH levels decline in the mid or late follicular phase. The expression of LH receptors in granulosa cells allows the larger follicles to grow and to develop dominance over smaller follicles [15].

The main functions of LH in the ovarian cycle include promoting steroid synthesis in granulosa cells acting in synergy with FSH, offering androgens as a substrate to oestradiol (E2) production, inducing the maturation of the oocyte up to the metaphase II state, inducing the production of proteases, and playing a special role in the ovulation. LH can also induce atresia of medium follicles when its concentration is greater than the “LH ceiling,” and finally it acts as an inductor of luteinization, namely the change that takes place in the structure and function of granulosa cells to produce progesterone and E2 during the luteal phase [16].

The action of LH and FSH is determined by a variety of factors: the frequency and amplitude of GnRH peaks, the different isoforms of LH and FSH, polymorphisms of FSH and LH and their receptors, and intracellular signalling. Furthermore, in OS for IVF, inter-individual demographic, clinical, and treatment factors, such as aging, comorbidities, and the effect of oral contraceptives and GnRH analogue protocols, can influence gonadotropin action and the response to exogenous gonadotropins [17].

Hypogonadotropic patients

The need for LH in the follicular phase is clearly demonstrated in hypogonadotropic patients. Hypogonadotropic hypogonadism (HH) is a rare reproductive function disorder characterized by the absence or decreased function of gonads due to a lack of effective hypothalamic–pituitary activity [18]. It results in arrested or attenuated gonadal function, and individuals with HH do not have the necessary threshold levels of endogenous LH required to achieve optimal follicular development and steroidogenesis after administration of FSH alone.

Congenital abnormalities are well-described rare conditions that usually present with deficient GnRH secretion occurring in isolation or in association with anosmia (Kallmann syndrome) [19, 20]. Among acquired conditions, intensive exercise and eating disorders are widely recognized as life-style factors that could suppress the hypothalamus–pituitary–gonadal (HPG) axis [21, 22]. Furthermore, poorly controlled diabetes and thyroid disorders could significantly affect gonadotropin secretion and action. To restore reproductive function, stress habits should be corrected, and the underlying endocrine disorder should be treated

[23, 24]. Other acquired conditions of reduced LH and FSH action could be linked to pituitary tumours (e.g. prolactinomas) or pituitary infarct (e.g. Sheehan's syndrome), which are usually characterized by specific symptoms due to pituitary dysfunction or compression of tissues surrounding the pituitary or that can severely affect pituitary function beyond reproductive function (e.g. panhypopituitarism) [25].

Patients with absence of endogenous gonadotropins are excellent models for studying the effects of LH. An open, randomized, dose-finding, multicentre study was designed with the aim of evaluating the efficacy of lutropin- α addition during follitropin- α stimulation, and of identifying the minimal effective dose in the treatment of women with HH. Thirty-eight women with HH and a mean age of 28.7 years received two daily subcutaneous injections of lutropin- α (0, 25, 75, or 225 IU) and follitropin- α (150 IU).

Analyses confirmed the strong influence of the recombinant LH (rLH) dose on E2 secretion, resulting in very different endometrial growth in the treatment groups. No pregnancies occurred in the 0- or 25-IU dose groups. In the 75- and 225-IU dose groups, pregnancy occurred in 16.6% and 11.1% of patients, respectively. Although the individual requirement of rLH varied, a daily dose of 75 IU rLH was effective in the majority of patients [26].

Use of LH in OS for IVF

In most cases, OS for IVF is performed under conditions of pituitary suppression to prevent LH surge and spontaneous ovulation through the use of GnRH analogues, either agonists or antagonists. Therefore, most patients reach very low concentrations of serum LH, similar to those observed in hypogonadotropic patients. The administration of LH activity in OS induces several differences in the synthesis of follicular steroids, which may have an impact on oocyte maturation and competence. On top of that, a combination of factors such as advanced maternal age and genetic variants of gonadotropins, or their receptors that impair gonadotropin action, may further exacerbate the transient reduced LH and FSH production caused by GnRH analogues, and result in a low or suboptimal response to OS [18].

To analyse the impacts of adding different amounts of rLH in OS on serum and follicular hormonal profiles, oocyte and embryo quality, and cycle outcomes, our group performed a randomized controlled trial in which 30 pure and altruistic norm-ovulatory oocyte donors aged 18–35 years, undergoing OS under

pituitary downregulation with a nafarelin long protocol, were allocated by computer-generated randomization to three groups [27]. Group A received 300 IU of recombinant FSH (rFSH) for starting OS. Group 2 received 225 IU of rFSH and 75 IU of rLH. Group 3 received 150 IU of rFSH and 150 IU of rLH. The initial protocol was maintained for two days. Then, serum E2 was determined and the rFSH dose adjusted, while rLH was continued with the same dose until the end of COS. When four or more follicles reached 18 mm in diameter, human chorionic gonadotropin (hCG) was administered and oocyte retrieval scheduled for 36 hours later.

On the day of hCG administration, serum E2, progesterone (P), androstenedione (A), testosterone (T), dehydroepiandrosterone sulphate (DHEAS), FSH, LH, and hCG were determined. The first two follicles of each ovary were aspirated individually, and E2, P, A, T, DHEAS, and LH were determined for each follicular fluid sample. Oocytes obtained from each follicle were labelled for classification and follow-up of the resulting embryo, if any.

The results of this study showed that, interestingly, no differences were observed among groups for any of the serum hormone determinations except for FSH levels, which were significantly higher in group A, as expected (Table 40.1). Figure 40.1 shows hormonal levels in follicular fluid. As can be observed, there was a dose-dependent increase of follicular fluid E2, A, and T according to LH dose. Metaphase I oocytes were obtained from follicles that had significantly lower E2 concentrations and higher T and A levels. On the other hand, oocytes that showed multiple anomalies were recovered from follicles with significantly higher LH levels. Oocytes with perivitelline space anomalies were obtained from follicles that showed significantly higher T, A, and DHEAS concentrations.

In summary, women who received high rLH amounts during OS produced follicles with higher androgens and E2 production. Defects of intrafollicular E2 are related to metaphase I oocytes, whereas excess of LH and androgens is related to oocyte anomalies.

The findings of steroids in follicular fluid are consistent with those observed in the MERIT study [28], in which patients who received highly purified human menopausal gonadotropin (hMG) for stimulation showed higher concentrations of E2, A, and T than those who were stimulated with rFSH. Therefore, the action of LH may be helpful for patients with low serum androgen levels.

It has been shown that serum androgens decline steeply with age, with a decrease from menarche to menopause that ranges

TABLE 40.1 Serum Hormone Concentrations the Day of Human Chorionic Gonadotropin Observation According to Different Amounts of Recombinant Follicle-Stimulating Hormone and Recombinant Luteinizing Hormone

	FSH 300 IU	FSH/LH 225/75 IU	FSH/LH 150/150 IU	P-value
E2 (pg/mL)	2662 ± 1239	2208 ± 852	2700 ± 1339	NS
P4 (ng/mL)	1.1 ± 0.7	0.6 ± 0.3	0.6 ± 0.5	NS
FSH (mIU/mL)	13.4 ± 4.5 (a)	8.6 ± 4.1 (b)	7.5 ± 1.3 (b)	0.009 (a > b)
LH (mIU/mL)	2.0 ± 1.9	1.6 ± 1.5	2.2 ± 1.8	NS
Te (ng/mL)	0.6 ± 0.2	0.5 ± 0.2	0.8 ± 0.3	NS
Δ4 (ng/mL)	2.7 ± 0.7	2.4 ± 0.4	2.9 ± 1.1	NS
DHEAS (μg/dL)	206 ± 57	190 ± 142	192 ± 78	NS

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, oestradiol; DHEAS, dehydroepiandrosterone sulphate; NS, non-significant; P4, progesterone; Te, testosterone; Δ4, androstenedione.

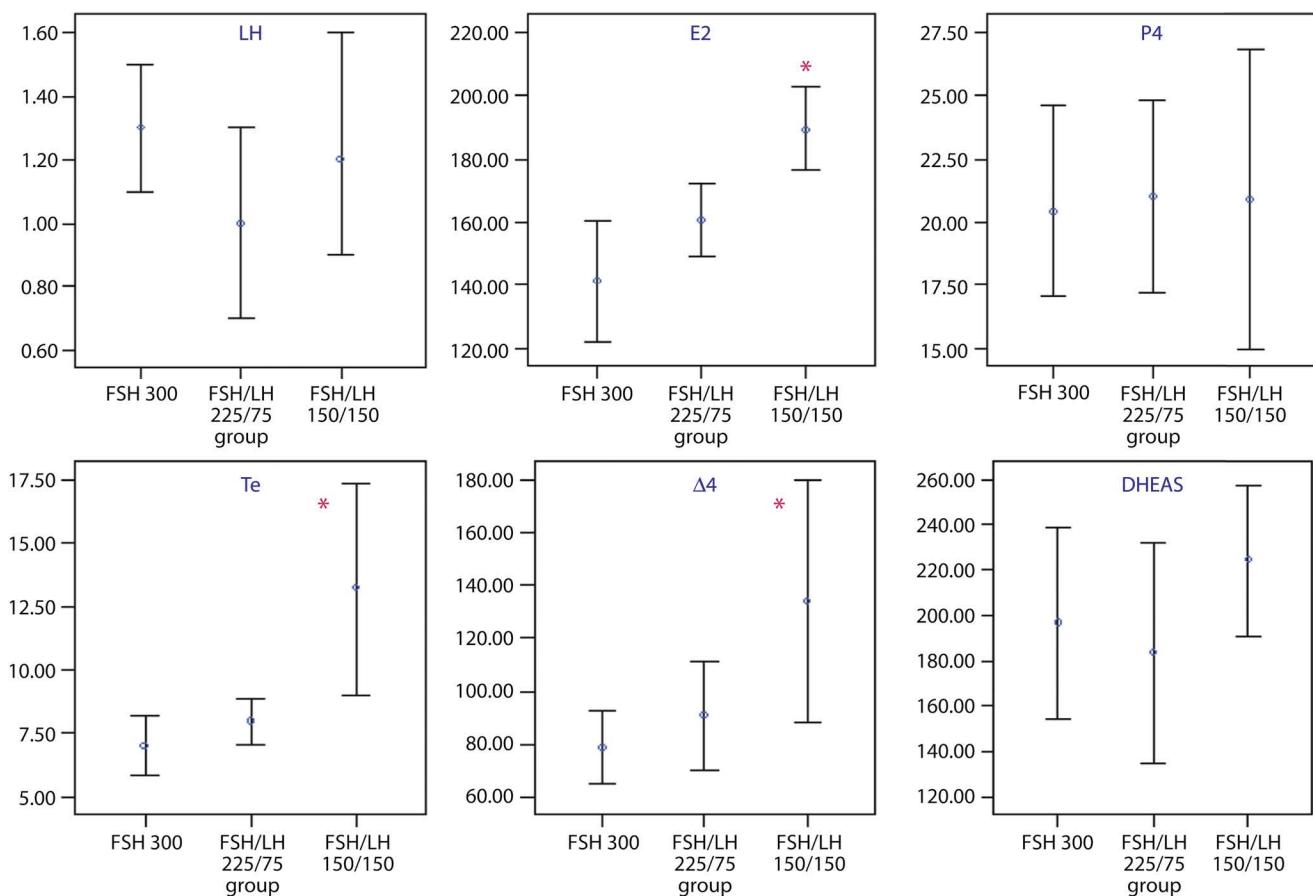


FIGURE 40.1 Follicular fluid hormonal determinations on the day of human chorionic gonadotropin observation. Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone (mIU/mL); E2, oestradiol (pg/mL); DHEAS, dehydroepiandrosterone sulphate (mcg/dL); P4, progesterone (ng/mL); Te, testosterone (ng/mL); Δ4, androstenedione (ng/mL). * $p < 0.05$.

from 49% for free T to 77% for DHEAS, despite constant levels of serum hormone binding globulin [29]. Moreover, it has been demonstrated that while the synthesis of E2 in response to rFSH stimulation is preserved in older women, there is a significant decrease in the synthesis of A in older women when rFSH alone is given for stimulation [30].

Indeed, in a prospective randomized study, we observed that in patients with basal T below the mean (0.45 ng/mL), the ongoing pregnancy rate was better when LH was associated with rFSH in COS for IVF, compared to rFSH alone in a GnRH agonist long protocol [31]. On the other hand, no differences were observed when both protocols were compared in women with T above the mean (Table 40.2). No other differences were observed with respect to other serum androgen levels. Taken together, this supports a potential benefit of LH administration in older women, for whom basal androgens and their synthesis in response to rFSH are diminished.

Normogonadotropic patients

This group includes the majority of patients that undergo ovarian stimulation for IVF. Studies published until now show that no benefit is obtained by combining LH and FSH in ovarian stimulation for IVF in normogonadotropic patients when using GnRHAs [5]. This is especially true for an unselected population [4].

Advanced reproductive age of women

The potential benefit of LH administration in patients of advanced reproductive age (i.e. >35 years) has been evaluated in a systematic review and meta-analysis [32]. In this study, it is clearly shown that LH administration leads to significantly better implantation and clinical pregnancy rates than rFSH-alone stimulation. Moreover, it is demonstrated that while rFSH leads to a higher oocyte yield, there are no differences in terms of metaphase II oocytes, and the fertilization rate is better in patients receiving LH.

These were also our findings in an age-adjusted randomized controlled trial performed in normogonadotropic patients following OS in a GnRH antagonist protocol [33]. It was observed that while results were virtually the same in both stimulation groups (rFSH vs rFSH + rLH) in patients aged up to 35 years, the implantation rate was significantly higher in women receiving rFSH and rLH in the 36–39 years of age group, with a clinically relevant increase in ongoing pregnancy rate.

Interestingly, serum progesterone levels at the end of stimulation were significantly higher in the rFSH group at all ages. This could be related to better endometrial receptivity when LH is given.

A similar randomized controlled trial has been published [34]. In it, patients aged 35 years or older were stimulated under

TABLE 40.2 Ongoing Pregnancy per Started Cycle According to Basal Androgen Levels

	FSH (95% CI)	FSH + LH (95% CI)	RR (95% CI)	P-value
Te ≤ 0.45 ng/mL	33.1 (25.4–41.7)	44.4 (36.1–53.2)	1.34 (0.98–1.85)	0.06
Te > 0.45 ng/mL	50.0 (37.5–62.5)	40.0 (28.6–52.6)	0.80 (0.53–1.20)	0.28
DHEAS ≤ 156 µg/L	32.4 (24.3–41.7)	38.2 (29.6–47.5)	1.18 (0.82–1.69)	0.37
DHEAS > 156 µg/L	47.3 (36.3–58.5)	43.4 (32.9–54.6)	0.92 (0.65–1.30)	0.63
Δ4 ≤ 1.90 ng/mL	39.1 (30.5–48.4)	46.0 (37.1–55.2)	1.18 (0.87–1.60)	0.30
Δ4 > 1.90 ng/mL	40.3 (29.7–51.8)	47.9 (36.9–59.2)	1.19 (0.82–1.72)	0.35

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; RR, relative risk; CI, confidence interval; DHEAS, dehydroepiandrosterone sulphate; Te, testosterone; Δ4, androstenedione.

a GnRH antagonist protocol and randomized to receive either rFSH alone across the cycle or to add 75 IU of rLH from day 6 of stimulation. In this study, no benefits of rLH administration were observed.

These findings could, at first glance, be contrary to those published by our group [33]. Nevertheless, an analysis in detail of the differences between both studies allows us to draw interesting and complementary conclusions about the possible role of LH in the treatment of this particular population [35]. Although the patients included in our study were of better prognosis (age limit 39 years and only first IVF cycles), the methodological differences that may explain the inconsistency of the results are the use of a contraceptive pill (CP) during the cycle prior to stimulation and the substitution of 75 IU of rFSH per day with 75 IU of rLH in the study group.

These differences are reflected in the ovarian response, in the synthesis of E2 and P, and in the follicular development and oocyte yield. Although in our study hormonal determinations before starting stimulation are not available, is very likely that after one cycle of CP, all values (E2, FSH, LH, P, and T) were lower than in the present study. This would explain the greater difficulty in response for the group receiving rFSH alone at the beginning of stimulation, due to excessive ovarian suppression. In this scenario, LH administration helps with better steroidogenesis due to greater androgen synthesis as a substrate for their later aromatization to oestrogens. This may also explain why in IVF cycles stimulated with rFSH alone and a GnRH antagonist, the administration of a CP during the previous cycle is associated with a lower pregnancy rate [36].

The substitution of 75 IU of rFSH with 75 IU of rLH from the beginning of stimulation may also explain the lower P levels on the day of hCG observation. Through its action at the theca layer, LH enhances the conversion of pregnenolone into androstenediol and A, while FSH enhances its conversion into progesterone in the granulosa cells. This progesterone cannot be converted into androgens in the human being [37], so if its production is excessive, it is delivered into circulation [38]. In fact, in a multivariate analysis of more than 4000 cycles, we observed that a P increase at the end of stimulation is significantly related to the daily dose of FSH, but not of LH [39].

So, the impact of LH on ovarian stimulation is more patent when its administration is started at the beginning of the cycle. In the König study, LH is given from the sixth day of stimulation, when follicular recruitment is already completed [34]. As a consequence, only a modest increase in E2 and T levels is observed, but this is probably too late to have an impact on the final response and cycle outcome. Indeed, no differences in terms of follicular response and oocyte yield are observed, whereas in our study,

patients who received LH obtained fewer overall oocytes, but more metaphase II oocytes, reflecting a selective role of LH in ovarian response [33]. This, together with lower P levels on the day of hCG observation, may explain the better outcome of these patients when rLH is administered from stimulation day 1.

Poor responders

Other authors have investigated the role of LH supplementation in patients who were hypo-responsive to ovarian stimulation with rFSH alone. These patients previously required very high doses of FSH (>3500 IU) or showed a plateau of follicular growth and E2 production when stimulated with FSH alone. These studies have shown that the addition of rLH during ovarian stimulation, when ovarian response to rFSH alone is adequate, leads to a better outcome than if the dose of FSH is increased [40–43]. The reason why some patients show this type of ovarian response may not be due merely to a low ovarian reserve. It has recently been suggested that the presence of a common LH polymorphism may explain the need for abnormally high amounts of rFSH for ovarian stimulation in IVF [44]. Although other studies have reported conflicting results [45, 46], meta-analysis from the Cochrane Database shows a clear benefit of LH administration in these types of patients [5]. On the other hand, a prospective randomized trial performed under the GnRH agonist long protocol didn't show any differences in terms of ovarian response or live birth rate between the stimulation with rFSH alone and with rFSH+rLH [47]. Nevertheless, a careful look at the results shows that in women aged until 40, live birth rates tend to be higher in the rFSH group, while in patients >40 live birth rates tend to be higher in the rFSH+rLH group.

Patients with high levels of LH

Polycystic ovary syndrome (PCOS) is a common endocrine disorder associated with obesity, hyperinsulinemia, elevated levels of androgens and LH, follicular atresia, and anovulation [48]. Furthermore, in PCOS, inappropriate pituitary gonadotropin secretion is generally characterized by higher mean LH serum concentrations, greater LH pulse frequency, and enhanced LH response to GnRH with respect to those of normal women [49]. LH acts on theca cells, increasing the secretion of androgens that induce atresia of non-dominant follicles [50]. Excessive LH secretion could be responsible for the abnormal follicle dynamics of PCOS patients, and may hasten late follicular-phase meiotic maturation [51].

While many studies which exclude PCOS have focused on the differences obtained with FSH compared to hMG [52], very few have been published about ovarian stimulation using gonadotropins in PCOS patients with LH activity. No differences were

found between outcomes in PCOS patients stimulated with rFSH versus those stimulated with hMG. Indeed, similar oocyte maturation and fertilization rates were achieved in both groups [53]. In a recent review about ovarian stimulation in women with PCOS, no significant difference was demonstrated between FSH and hMG in terms of pregnancy rate. However, given the potential advantages in terms of purity and a reduction in the risk of ovarian hyperstimulation syndrome (OHSS), highly purified FSH or rFSH are likely to be widely adopted in the future [54].

In a study of 20 patients with PCOS, 10 received hMG and 10 were stimulated with FSH, with a reduction of DHEAS synthesis being observed in the former group. These findings suggest that, in PCOS patients, exogenous hMG induces a different steroid synthesis pattern than pure FSH, possibly by reduction of the $\delta 5$ steroid synthesis pathway in the adrenals and/or in the ovary [55].

There is a fear surrounding the use of gonadotropins with LH activity in PCOS patients because of the risk of OHSS, but no prospective study has yet demonstrated that the use of LH increases this risk in said patients.

Insulin seems to modulate LH levels, as has been recently reported in a study showing a clear alteration of LH levels as a direct result of insulin infusion [56]. Drugs that exert an action on insulin resistance in PCOS patients have been extensively described, particularly metformin, but this topic is beyond the scope of this review.

In summary, on the basis of the available evidence, it is not possible to confirm the benefits and the harmful effects of gonadotropins on LH in PCOS patients. A well-designed study is required to answer the question of whether LH is necessary in women with PCOS.

Conclusion

The treatment of infertility involves OS, which often calls for the use of gonadotropins. Both FSH and LH form part of the therapeutic arsenal employed to achieve multiple follicular development. The need to develop protocols that improve the possibility of infertile patients becoming parents is a major challenge to both clinicians and pharmaceutical companies. In the next few years, it will be crucial to clearly define the differences in ovarian response, oocyte–embryo quality, endometrial receptivity, and cycle outcomes between patients undergoing IVF–embryo transfer with a combination of rFSH and rLH and those receiving the more established rFSH and hMG protocols.

Studies have provided sufficient evidence to support the proposed dose of 75 IU of lutropin- α in the combined product, and, in general terms, a starting dose of 75 IU would appear to be appropriate.

Studies reveal that while young, normovulatory patients do not benefit from the use of rLH, there is a specific population in which better results are achieved when rLH is combined with rFSH. Although a clear definition of such patients is still lacking, the available data suggest that some women over 35 years of age, and women with hypo-responsiveness to FSH that may be carriers of gonadotrophin receptor polymorphisms may benefit from rLH administration.

Also, a supplementation of rLH to rFSH in a 1:2 ratio has been shown to lower the risk of suffering an increase of P levels at the end of stimulation [57].

Finally, it seems that LH can provide a means of selecting larger follicles and curtailing smaller, less mature follicles, and it can be used to rescue the luteal phase in patients in whom ovulation

induction is performed with a GnRH agonist, a strategy that is used to prevent OHSS [58].

References

- Bosch E, Ezcurra D. Individualised controlled ovarian stimulation (iCOS): Maximising success rates for assisted reproductive technology patients. *Reprod Biol Endocrinol*. 2011;9:82.
- Balasch J, Miró F, Burzaco I, Casamitjana R, Civico S, Ballesca JL, Puerto B, vanrell JA. The role of luteinizing hormone in human follicle development and oocyte fertility: Evidence from IVF in a woman with long-standing hypogonadism and using recombinant human follicle stimulating hormone. *Hum Reprod*. 1995;10:1678–83.
- Recombinant human luteinizing hormone (LH) to support recombinant human follicle-stimulating hormone (FSH)-induced follicular development in LH- and FSH-deficient anovulatory women: A dose-finding study. The European Recombinant Human LH Study Group. *J Clin Endocrinol Metab*. 1998;83:1507–14.
- Kolibianakis EM, Kalogeropoulou L, Griesinger G, Papanikolaou EG, Papadimas J, Bontis J, Tarlatzis BC. Among patients treated with FSH and GnRH analogues for *in vitro* fertilization, is the addition of recombinant LH associated with the probability of live birth? A systematic review and meta-analysis. *Hum Reprod Update*. 2007;13:445–52.
- Mochtar MH, Van der V, Ziech M, van Wely M. Recombinant luteinizing hormone (rLH) for controlled ovarian hyperstimulation in assisted reproductive cycles. *Cochrane Database Syst Rev*. 2007;2:CD005070.
- Lamminen T, Jokinen P, Jiang M, Pakarinen P, Simonsen H, Huhtaniemi I. Human FSH β subunit gene is highly conserved. *Mol Hum Reprod*. 2005;11:601–5.
- Fauser BC, Van Heusden AM. Manipulation of human ovarian function: Physiological concepts and clinical consequences. *Endocr Rev*. 1997;18:71–106.
- Brailly S, Gougeon A, Milgrom E, Bomsel HO, Papiernik E. Androgens and progestins in the human ovarian follicle: Differences in the evolution of preovulatory, healthy nonovulatory, and atretic follicles. *J Clin Endocrinol Metab*. 1981;53:128–34.
- Loumaye E. Ovarian stimulation: Is exogenous LH necessary in all patients? *Gynecol Obstet Fertil*. 2002;30:890–5.
- Chappel SC, Howles C. Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. *Hum Reprod*. 1991;6:1206–12.
- Fritz MA, Speroff L (Eds.). The ovary—Embryology and development. In: *Clinical Endocrinology and Infertility*. Philadelphia, PA: Lippincott, Williams and Wilkins, pp. 105–20, 2011.
- Kornyei JL, Lei ZM, Rao CHV. Human myometrial smooth muscle cells are novel targets of direct regulation by human chorionic gonadotropin. *Biol Reprod*. 1993;49:1149–57.
- Nordhoff V, Gromoll J, Simoni M. Constitutively active mutations of G protein-coupled receptors: The case of human luteinizing hormone and follicle-stimulating receptors. *Arch Med Res*. 1999;30:501–9.
- Wide L, Bakos O. More basic forms of both human follicle stimulating hormone and luteinizing hormone in serum at mid-cycle compared with follicular or luteal phase. *J Clin Endocrinol Metab*. 1993;76:885–9.
- Campbell BK, Dobson H, Baird DT, Scaramuzzi RJ. Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle selection in sheep. *J Reprod Fertil*. 1999;117:355–67.
- Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*. 2001;122:829–38.
- Bosch E, Alviggi C, Lispi M, Conforti A, Hanyaloglu AC, Chuderland D, Simoni M, Raine-Fenning N, Crépieux P, Kol S, Rochira V, D'Hooghe T, Humaidan P. Reduced FSH and LH action: Implications for medically assisted reproduction. *Hum Reprod*. 2021;36:1469–80.

18. Reame NE, Sauder ED, Case GD, Kelch RP, Marshall JC. Pulsatile gonadotropin secretion in women with hypothalamic amenorrhea: Evidence that reduced frequency of gonadotropin releasing hormone secretion is the mechanism of persistent anovulation. *J Clin Endocrinol Metab.* 1985;61:851–8.
19. Kallmann FJ, Schoenfeld WA, Barrera SE. The genetic aspects of primary eunuchoidism. *Am J Ment Def.* 1944;48:203–36.
20. Boehm U, Bouloux P-M, Dattani MT, N de R, Dode' C, Dunkel L, Dwyer AA, Giacobini P, Hardelin J-P, Juul A, et al. Expert consensus document: European Consensus statement on congenital hypogonadotropic hypogonadism—pathogenesis, diagnosis and treatment. *Nat Rev Endocrinol.* 2015;11:547–64.
21. Boyar RM, Katz J, Finkelstein JW, Kapen S, Weiner H, Weitzman ED, Hellman L. Anorexia nervosa. Immaturity of the 24-hour luteinizing hormone secretory pattern. *N Engl J Med.* 1974;291:861–5.
22. Shangold MM. Causes, evaluation, and management of athletic oligo-/amenorrhea. *Med Clin North Am.* 1985;69:83–95.
23. Strotmeyer ES, Steenkiste AR, Foley TP, Berga SL, Dorman JS. Menstrual cycle differences between women with type 1 diabetes and women without diabetes. *Diabetes Care.* 2003;26:1016–21.
24. Gordon CM. Clinical practice. Functional hypothalamic amenorrhea. *N Engl J Med.* 2010;363:365–71.
25. Li RHW, Ng EHY. Management of anovulatory infertility. *Best Pract Res Clin Obstet Gynaecol.* 2012;26:757–68.
26. Bosch E. Recombinant human follicular stimulating hormone and recombinant human luteinizing hormone in a 2:1 ratio combination. Pharmacological characteristics and clinical applications. *Expert Opin Biol Ther.* 2010;10:1001–9.
27. Bosch Pau E, Albert C, Zuzuarregui JL, Remohí J, Pellicer A. Impact of different amounts of LH in controlled ovarian hyperstimulation oocyte donation cycles. *Fertil Steril.* 2006;86(Suppl 2):S425.
28. Smitz J, Andersen AN, Devroey P, Arce JCMERIT Group. Endocrine profile in serum and follicular fluid differs after ovarian stimulation with HP-hMG or recombinant FSH in IVF patients. *Hum Reprod.* 2007;22:676–87.
29. Davison SL, Bell R, Donath S, Montalto JG, Davis SR. Androgen levels in adult females: Changes with age, menopause, and oophorectomy. *J Clin Endocrinol Metab.* 2005;90:3847–53.
30. Welt CK, Jimenez Y, Sluss PM, Smith PC, Hall JE. Control of estradiol secretion in reproductive ageing. *Hum Reprod.* 2006;21:2189–93.
31. Bosch E, Labarta E, Vidal C, Giles J, Bellver J, Zuzuarregui JL, Pellicer A. The relationship between serum androgen levels and the need of LH administration during controlled ovarian stimulation for *in vitro* fertilization: An explorative study. *Hum Reprod.* 2011;26(Suppl 1):i26O: 064.
32. Hill MJ, Levens ED, Levy G, Ryan ME, Csokmay JM, DeCherney AH, Whitcomb BW. The use of recombinant luteinizing hormone in patients undergoing assisted reproductive techniques with advanced reproductive age: A systematic review and meta analysis. *Fertil Steril.* 2012;97:1108–14.
33. Bosch E, Labarta E, Crespo J, Simón C, Remohí J, Pellicer A. Impact of luteinizing hormone administration on gonadotropin-releasing hormone antagonist cycles: An age-adjusted analysis. *Fertil Steril.* 2011;95:1031–6.
34. König TE, van der Houwen LE, Overbeek A, et al. Recombinant LH supplementation to a standard GnRH antagonist protocol in women of 35 years or older undergoing IVF/ICSI: A randomized controlled multicentre study. *Hum Reprod.* 2013;28:2804–12.
35. Bosch E. Comment on “Recombinant LH supplementation to a standard GnRH antagonist protocol in women of 35 years old or older undergoing IVF/ICSI: A randomized controlled multicentre study”. *Hum Reprod.* 2014;29:636–7.
36. Griesinger G, Kolibianakis EM, Venetis C, Diedrich K, Tarlatzis B. Oral contraceptive pretreatments significantly reduces ongoing pregnancy likelihood in gonadotropin-releasing hormone antagonist cycles: An updated meta-analysis. *Fertil Steril.* 2010;94:2382–4.
37. Yding Andersen C, Bungum L, Nyboe Andersen A, Humaidan P. Preovulatory progesterone concentration associates significantly to follicle number and LH concentration but not to pregnancy rate. *Reprod Biomed Online.* 2011;23:187–95.
38. Fleming R, Jenkins J. The source and implications of progesterone rise during the follicular phase of assisted reproduction cycles. *Reprod Biomed Online.* 2010;21:446–9.
39. Bosch E, Labarta E, Crespo J, Simón C, Remohí J, Jenkins J, Pellicer A. Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for *in vitro* fertilization: Analysis of over 4000 cycles. *Hum Reprod.* 2010;25:2092–100.
40. Lisi F, Rinaldi L, Fishel S, Lisi R, Pepe GP, Picconeri MG. Use of recombinant follicle-stimulating hormone (Gonal F) and a recombinant luteinizing hormone (Luveris) in multiple follicular stimulation in patients with a suboptimal response to *in vitro* fertilization. *Fertil Steril.* 2003;79:1037–8.
41. Ferraretti AP, Gianaroli L, Magli MC, D'Angelo A, Farfalli V, Montanaro N. Exogenous luteinizing hormone in controlled ovarian hyperstimulation for assisted reproduction techniques. *Fertil Steril.* 2004;82:1521–6.
42. De Placido G, Alviggi C, Perino A, Strina I, Lisi F, Fasolino A, Italian Collaborative Group on Recombinant Human Luteinizing Hormone. Recombinant human LH supplementation versus recombinant human FSH (rFSH) step-up protocol during controlled ovarian stimulation in normogonadotropic women with initial inadequate ovarian response to rFSH. A multicentre, prospective, randomized controlled trial. *Hum Reprod.* 2005;20:390–6.
43. Ruvolo G, Bosco L, Pane A, Morici G, Cittadini E, Roccheri MC. Lower apoptosis rate in human cumulus cells after administration of recombinant luteinizing hormone to women undergoing ovarian stimulation for *in vitro* fertilization procedures. *Fertil Steril.* 2007;87:542–6.
44. Alviggi C, Clarizia R, Pettersson K, Mollo A, Humaidan P, Strina I, Coppola M, Ranieri A, D'Uva M, De Placido G. Suboptimal response to GnRHa long protocol is associated with a common LH polymorphism. *Reprod Biomed Online.* 2009;18:9–14.
45. Chung K, Krey L, Katz J, Noyes N. Evaluating the role of exogenous luteinizing hormone in poor responders undergoing *in vitro* fertilization with gonadotropin-releasing hormone antagonist. *Fertil Steril.* 2005;84:313–8.
46. Barrenetxea G, Agirrekoiko JA, Jiménez MR, de Larruzea AL, Ganzabal T, Carbonero K. Ovarian response and pregnancy outcome in poor-responder women: A randomized controlled trial on the effect of luteinizing hormone supplementation on *in vitro* fertilization cycles. *Fertil Steril.* 2008;89:546–53.
47. Humaidan P, Chin W, Rogoff D, D'Hooghe T, Longobardi S, Hubbard J, Schertz J, ESPART Study Investigators‡. Efficacy and safety of follitropin alfa/lutropin alfa in ART: A randomized controlled trial in poor ovarian responders. *Hum Reprod.* 2017;32:544–55.
48. Vignesh JP, Mohan V. Polycystic ovary syndrome: A component of metabolic syndrome? *J Postgrad Med.* 2007;53:128–34.
49. The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod.* 2004;19:41–7.
50. Uilenbroek JT, Woutersen PJ, van-der Schoot P. Atresia of preovulatory follicles: Gonadotropin binding and steroidogenic activity. *Biol Reprod.* 1980;23:219–29.
51. Filicori M, Cognigni G, Samara A, Melappioni S, Perri T, Cantelli B, Parmegiani L, Pelusi G, DeAloysio D. The use of LH activity to drive folliculogenesis: Exploring uncharted territories in ovulation induction. *Hum Reprod Update.* 2002;8:543–57.
52. Coomarasamy A, Afnan M, Cheema D, van der Veen F, Bossuyt PMM, van Wely M. Urinary hMG versus recombinant FSH for controlled ovarian hyperstimulation following an agonist long down-regulation protocol in IVF or ICSI treatment: A systematic review and meta-analysis. *Hum Reprod.* 2008;23:310–5.

53. Teissier MP, Chable H, Paulhac S, Aubard Y. Recombinant human follicle stimulating hormone versus human menopausal gonadotropin induction: Effects in mature follicle endocrinology. *Hum Reprod.* 1999;14:2236–41.
54. Weiss NS, Nahuis M, Bayram N, Mol BWJ, Van der Veen F, van Wely M. Gonadotrophins for ovulation induction in women with polycystic ovarian syndrome. *Cochrane Database Syst Rev.* 2015;9:CD010290.
55. Turkmen S, Backstrom T, Idil M. Reduction of dehydroepiandrosterone sulphate synthesis in women with polycystic ovary syndrome by human menopausal gonadotropin but not purified urinary follicle stimulating hormone: A comparative pilot study. *Gynecol Endocrinol.* 2004;19:69–78.
56. Lawson MA, Jain S, Sun S, Patel K, Malcolm PJ, Chang RJ. Evidence for insulin suppression of baseline luteinizing hormone in women with polycystic ovarian syndrome and normal women. *J Clin Endocrinol Metab.* 2008;93:2089–96.
57. Werner MD, Forman EJ, Hong KH, Fransasiak JM, Molinaro TA, Scott RT Jr. Defining the “sweet spot” for administered luteinizing hormone-to-follicle stimulating hormone gonadotropin ratios during ovarian stimulation to protect against a clinically significant late follicular increase in progesterone: An analysis of 10,280 first *in vitro* fertilization cycles. *Fertil Steril.* 2014;102:1312–7.
58. Papanikolaou EG, Verpoest W, Fatemi H, Tarlatzis B, Devroey P, Tournaye H. A novel method of luteal supplementation with recombinant luteinizing hormone when a gonadotropin-releasing hormone agonist is used instead of human chorionic gonadotropin for ovulation triggering: A randomized prospective proof of concept study. *Fertil Steril.* 2011;95:1174–7.

ENDOCRINE CHARACTERISTICS OF ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES

Bulent Urman, Baris Ata, and Hakan Yarali

Introduction

Ovarian stimulation (OS) for assisted reproductive technology (ART) cycles aims to provide multiple pre-ovulatory follicles available for oocyte collection. There are three main components of a conventional ART stimulation cycle: (i) induction of multi-follicular growth with exogenous gonadotropins, (ii) prevention of premature ovulation before oocyte collection through suppression of endogenous luteinizing hormone (LH) surge, and (iii) induction of an endogenous LH surge or mimicking it with exogenous human chorionic gonadotropin (hCG) for oocyte maturation. In this chapter, we will briefly review endocrinologic aspects of each of these components.

Induction of multi-follicular growth with exogenous gonadotropins

A finite number of primordial follicles exist in the ovaries of reproductive aged women. A cohort of these primordial follicles starts growing in a random and continuous fashion; a process called “primary recruitment.” Primordial follicle growth occurs until the antral stage independent of gonadotropin stimulation. For further growth, follicle stimulating hormone (FSH) is required. In the absence of adequate FSH supply, as happens before puberty, antral follicles undergo atresia before reaching the pre-ovulatory stage. FSH threshold is the minimum level of FSH required for continuing follicle growth beyond the antral stage. Importantly, follicles at different stages of growth have different FSH thresholds, a fact that precludes defining it with a single serum FSH level.

In a natural cycle, during the luteo-follicular transition, endogenous FSH production increases following the demise of the corpus luteum and the resultant fall in progesterone, oestradiol, and inhibin A levels. When the increasing FSH level exceeds the threshold, it enables the antral follicles, which have gained FSH responsiveness through expression of FSH receptors on the granulosa cells, to continue growth, a process that is called “secondary” or “cyclic, gonadotropin dependent recruitment” [1]. The number of antral follicles recruited during the luteo-follicular transition is proportional to ovarian reserve, i.e. the number of resting primordial follicles, in the ovaries.

Growing antral follicles produce increasing amounts of oestradiol and inhibin B, which exert negative feedback to the hypothalamus and pituitary, leading to a decline in pituitary FSH production to levels below the threshold. While the antral follicles, that are still dependent on FSH for growth, undergo atresia, the dominant follicle that has started expressing LH receptors on its granulosa cells can continue its growth despite declining FSH levels. The period of FSH supply over the threshold is named the “FSH window” (Figure 41.1). The rationale of OS for ART is to increase the number of follicles reaching the pre-ovulatory

stage, a process that requires extension of the FSH window. This is achieved either by exogenous FSH administration or by anti-estrogenic agents that block the negative feedback mechanisms, i.e. selective oestrogen receptor modulators or aromatase inhibitors.

In conventional ART cycles, exogenous FSH administration is started in the early follicular phase, a period where endogenous FSH levels fall below the threshold for the already existing antral follicles. This enables the growth of a group of antral follicles up to the pre-ovulatory stage. Follicular response to FSH stimulation is monitored by ultrasound examination of the ovaries, and serum oestradiol measurements provide a rough estimate of follicular growth during ovarian stimulation. While serum oestradiol levels <100 pg/mL on the sixth day of FSH stimulation suggest an inadequate follicular response, levels >500 pg/mL have been considered a sign of overstimulation. However, what extent of follicular growth represents overstimulation is unclear in an era when cumulative live birth rate or family completion rate per stimulation cycle is regarded as a measure of successful ovarian stimulation for ART [2]. The introduction of GnRH agonist trigger coupled with increased success of vitrification have brought about the concept of “elective freeze all” with postponement of fresh embryo transfer, and curbed the risk of ovarian hyperstimulation syndrome (OHSS). This also helps to avoid adverse effects of increased sex steroid levels on the endometrium that may decrease the success of fresh embryo transfer [3]. Regardless, the course of serum oestrogen levels reflects follicular growth throughout stimulation. Inhibin B is another product of granulosa cells of early antral follicles and its serum levels can also be used as a marker of follicular growth. Indeed, earlier studies demonstrated an association between serum inhibin B levels between the fourth and sixth day of FSH stimulation and the number of mature oocytes collected. However, additional value of measuring inhibin B levels over ultrasound and serum oestradiol monitoring is questionable, and not routinely practiced.

The current paradigm suggests that serum FSH levels are not informative with regard to follicular growth; thus, FSH levels are not monitored routinely. The most likely reason is the limiting factor for follicular response being the number of available antral follicles rather than FSH level per se, provided that FSH is above the threshold. Moreover, threshold varies for individual follicles, and there's a significant overlap in serum FSH levels between anovulatory women who responded with or without follicular growth to exogenous FSH stimulation [4]. There is an ongoing debate on the value of individualization of starting FSH dosage and/or dose adjustments during stimulation based on markers of ovarian response [5, 6]. Circulating FSH concentration is the sum of exogenously administered FSH and endogenous FSH. Pharmacokinetics of exogenous FSH and effects of increasing levels of inhibin B along with pituitary suppression on endogenous FSH levels can vary across individuals [7]. Therefore, more studies

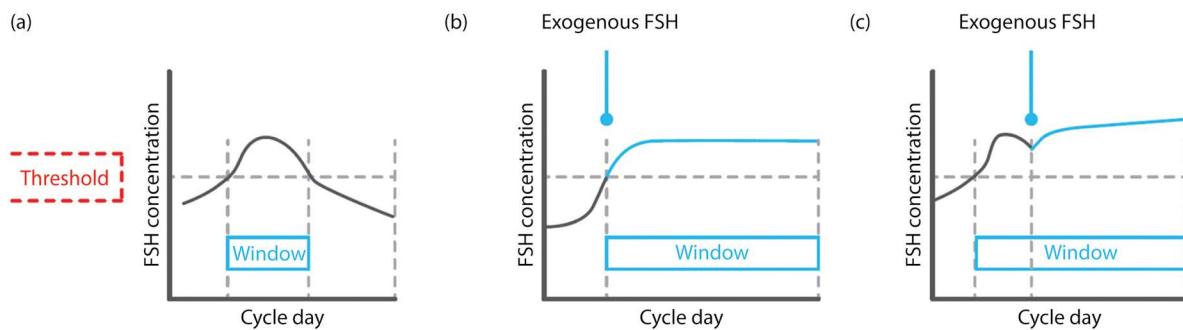


FIGURE 41.1 The FSH window corresponds to the period during which FSH levels are above the threshold levels for continuing antral follicle growth. (a) Natural menstrual cycle; (b) long GnRH agonist protocol; (c) GnRH antagonist cycle. Abbreviation: FSH, follicle-stimulating hormone.

are required to understand whether serum FSH levels during stimulation can be used to guide these decisions.

Prevention of endogenous luteinizing hormone surge and follicle rupture

Multi-follicular growth induced with exogenous FSH stimulation risks a premature LH surge, which can lead to the rupture of follicles before oocyte collection. This is prevented by blocking GnRH action on the pituitary gonadotrophs. GnRH analogues are commonly used to achieve pituitary suppression in two different ways. The first involves pituitary desensitization by a prolonged exposure to exogenous GnRH, i.e. GnRH agonist administration starting from the mid-luteal phase of the preceding cycle or simultaneously with gonadotropin injections, the long luteal GnRH agonist and the short GnRH agonist protocols, respectively. The second involves daily administration of a GnRH antagonist when an endogenous LH surge is likely to occur, i.e. in the late follicular phase. GnRH antagonists compete with endogenous GnRH at the receptor level and provide rapid blockage of GnRH activity.

GnRH agonist injections initially lead to the release of FSH and LH from the pituitary, i.e. a flare effect, but they eventually provide a hypogonadotropic state, i.e. severely suppressed endogenous FSH and LH production. This is due to internalization of GnRH receptors on the gonadotrophs following prolonged exposure to GnRH. To the contrary, in GnRH antagonist protocols, endogenous gonadotropin production remains unaltered until the initiation of GnRH antagonist in the late follicular phase. Thus, overall FSH consumption is lower in GnRH antagonist cycles than in GnRH agonist cycles.

Progesterins have recently emerged as an alternative to GnRH analogues for pituitary suppression. The use of progestins for pituitary suppression during OS is called progestin primed ovarian stimulation (PPOS). The exact mechanism of pituitary suppression by progestins is unclear. While progesterone can be a facilitator of endogenous LH surge at the end of follicular phase in natural cycles, higher dosages or prolonged exposure block the LH surge. The interactions between progesterone, kisspeptin, GnRH, gonadotropins, oestradiol, other molecules, and their time dependency for triggering or blocking LH surge remain to be identified. However, micronized progesterone and a variety of progestins, in various dosages have been successfully used to

prevent premature ovulation in OS cycles when fresh transfer is not contemplated [8]. Compared with GnRH analogues, PPOS yields a similar number of oocytes with comparable gonadotropin consumption. Furthermore, studies of PPOS, random start, or luteal phase ovarian stimulation cycles report reassuring results for safety of exposure of developing follicles to elevated progesterone levels regarding embryo development and euploidy rates [8, 9].

The role of luteinizing hormone

The two-cell, two-gonadotropin theory suggests that ovarian steroidogenesis is the result of actions of FSH and LH on granulosa and theca cells, respectively, through receptors specific to each gonadotropin. LH stimulates conversion of cholesterol to androstenedione in theca cells. Androstenedione diffuses into the granulosa cells, where, under the influence of FSH, is aromatized to oestrogens. Thus, LH action is necessary for production of oestradiol. In studies conducted on hypogonadal subjects, stimulation by only FSH promotes follicle development but cannot induce steroidogenesis [10].

The concept of a therapeutic LH window that has been introduced by Balasch and Fabreques states that below a certain threshold of LH, follicular maturation is impaired due to inadequate theca cell androgen synthesis and reduced aromatization of androgens to oestrogens resulting in incomplete oocyte maturation [11]. If serum LH level is kept in an optimum range, follicular growth and development leads to full oocyte maturation. GnRH analogues used during ovarian stimulation create an LH deficient environment that may, in theory, be detrimental to follicle growth and maturity [12]. Abnormally high levels of LH on the other hand result in LH receptor downregulation, enhanced intraovarian androgen production and impaired granulosa cell proliferation leading to atresia of the subordinate follicles and premature luteinization of the dominant follicle [11]. LH may also play a role in the deselection of subordinate follicles. Preclinical evidence showed that developing follicles have specific requirements for exposure to LH beyond which normal maturation ceases [13]. This finding gave rise to the concept of an “LH ceiling,” meaning that each follicle would have an upper limit of stimulation.

LH also acts on the granulosa cells through its own receptors. Therefore, it appears that LH regulates both granulosa and theca cells during the late follicular phase. FSH and LH induce the local

production of the soluble molecule inhibin B and growth factors. Among these, insulin-like growth factors (IGF) I and II, which are expressed by both granulosa and theca cells throughout folliculogenesis, are important in promoting follicular maturation [14]. These findings may explain the observation that FSH activity can be totally substituted by LH during the late follicular phase once granulosa cells express adequate amounts of LH receptors [15].

Besides its role in follicle growth and maturation, the secretion of LH may, in theory, be beneficial in reducing the exposure of the growing follicles and the endometrium to a subtle increase in progesterone concentrations. The relevance of late follicular phase progesterone concentrations will be discussed later. It may be concluded that LH is necessary for optimal follicular growth, steroid environment, and implantation. However, whether too much LH is detrimental for follicular growth, retrieval of good quality mature oocytes, and embryo implantation is still a matter of debate.

Despite these theoretical concerns, findings from clinical trials of LH supplementation of ovarian stimulation are conflicting. Currently, three groups of commercially available gonadotropin preparations contain LH activity: (i) urinary human menopausal gonadotropins (hMG), in which 95% of the LH activity is derived from hCG; (ii) LH glycoprotein produced by recombinant technology, and (iii) a combination of recombinant FSH and LH glycoproteins in a fixed ratio of 2:1.

Retrospective evaluation of a large number of randomized controlled trials (RCTs) comparing recombinant FSH (rFSH) with HMG or corifollitropin alpha, an extended action FSH molecule, failed to show any association between endogenous LH levels and ART outcome [16, 17]. It appears that low endogenous LH levels associated with the long luteal GnRH agonist protocol do not decrease the probability of a successful ART outcome.

It is generally concluded that ample evidence exists for the equivalence of rFSH and HMG regarding clinical outcome of ART cycles [2, 18]. However, given the higher oocyte yield in the rFSH group, more RCTs are required to compare the cumulative pregnancy rates, including fresh and frozen-thawed embryo transfers from one stimulation cycle. Despite the fact that rFSH stimulates more follicles resulting in higher peak oestradiol levels and is associated with a higher number of retrieved oocytes, it appears that the incidence of OHSS is similar to those women stimulated with urinary gonadotropins [19]. There also does not appear to be a difference in pregnancy rates of frozen thawed embryo transfer cycles that were previously treated with rFSH or HMG [20].

The addition of rLH to rFSH or the combination preparations of rLH and rFSH have been compared with rFSH, in several studies. The endocrine profile of ART cycles stimulated with rFSH and rLH versus HMG were compared in a prospective study involving oocyte donors [21]. On the sixth day of stimulation and on the day of triggering, serum steroid hormone levels were slightly but not significantly higher in the rFSH group compared with the HMG group. No statistically significant differences were observed for intrafollicular levels of steroid hormones between the two protocols; ongoing pregnancy rates were also similar (46.1% vs 46.1%). It appears that the endocrine profile of the COS cycle is not affected by the source of LH activity.

In conclusion, while LH is absolutely required for women with hypogonadotropic hypogonadism (WHO Group I anovulation), there's inadequate evidence to prove that routine LH administration to other women is associated with an improvement in ART outcome, including implantation and pregnancy rates [22]. However, there is some evidence suggesting a beneficial effect of LH in subsets of patients, perhaps older women [23].

Given the aforementioned uncertainties, routine monitoring of serum LH levels, to confirm pituitary downregulation in the long GnRH agonist protocol or to determine an endogenous LH surge or to identify women requiring LH supplementation, seems unwarranted during stimulation cycles. Plasma LH levels rapidly decline after GnRH antagonist administration and the relevance of an LH surge without an accompanying increase in progesterone level is controversial. Therefore, the detection of an isolated LH surge in GnRH antagonist stimulation cycles is unlikely to alter management of the cycle.

Early follicular phase progesterone levels

Menstrual bleeding follows the demise of the corpus luteum, which is the source of progesterone. In a natural cycle, serum progesterone levels are <1 ng/mL until the start of an LH surge. In the long GnRH agonist protocol, corpus luteum can be rescued by the initial flare effect of the GnRH agonist on LH secretion. Increased progesterone levels accompanied by the presence of an ovarian cyst on the starting day of gonadotropin injections suggests the presence of an active corpus luteum. This would require extending downregulation with the GnRH agonist and delaying gonadotropin start until after demise of corpus luteum. However, routine measurement of serum progesterone levels to confirm pituitary downregulation is not required if the ultrasound scan shows a thin endometrium in the absence of a follicle >10 mm in size.

Incomplete luteolysis is the most likely reason for high progesterone levels early in the cycle. Serum progesterone level above 1.5 ng/mL on the second day of a spontaneous menstrual cycle has been reported in 4%–13% of women who were due to start ovarian stimulation in a GnRH antagonist cycle [24–26]. Studies have consistently shown significantly decreased pregnancy rates in women with elevated early follicular phase progesterone levels. However, given the low incidence of elevated progesterone on the second day of the cycle, and the absence of a proven intervention to restore pregnancy rates, routine screening of serum progesterone levels before commencing stimulation is not recommended.

Late follicular phase progesterone elevation during ovarian stimulation for IVF

Late follicular phase progesterone elevation (LFPE) occurs in up to 46.7% of fresh ART cycles; however, its impact on the reproductive outcomes is still controversial [27]. In the early follicular phase, the adrenal gland constitutes the main source of progesterone, whereas the progesterone production shifts towards the ovaries during the late follicular phase [28, 29]. Several mechanisms have been proposed to explain supra-physiological LFPE. Since LFPE is not hindered by the use of GnRH analogues, the traditional concept that LFPE is produced both by the theca and granulosa cells in response to endogenous LH (premature luteinization) has been challenged [30]. Recent evidence shows that LFPE is probably due to excess production of progesterone in the granulosa cells, mediated by FSH activity in a dose-dependent manner [31]. FSH directly stimulates the expression of 3-beta-hydroxysteroid dehydrogenase in the granulosa cells to increase the conversion of pregnenolone into progesterone in a dose-dependent fashion. However, 17-alpha-hydroxylase activity is only present in the theca cells and lacks in the granulosa cells. Hence progesterone produced by the granulosa cells either diffuses into the theca cells to be metabolized (hydroxylated) or may acquire access to the circulation if produced in excess amounts (Figure 41.2).

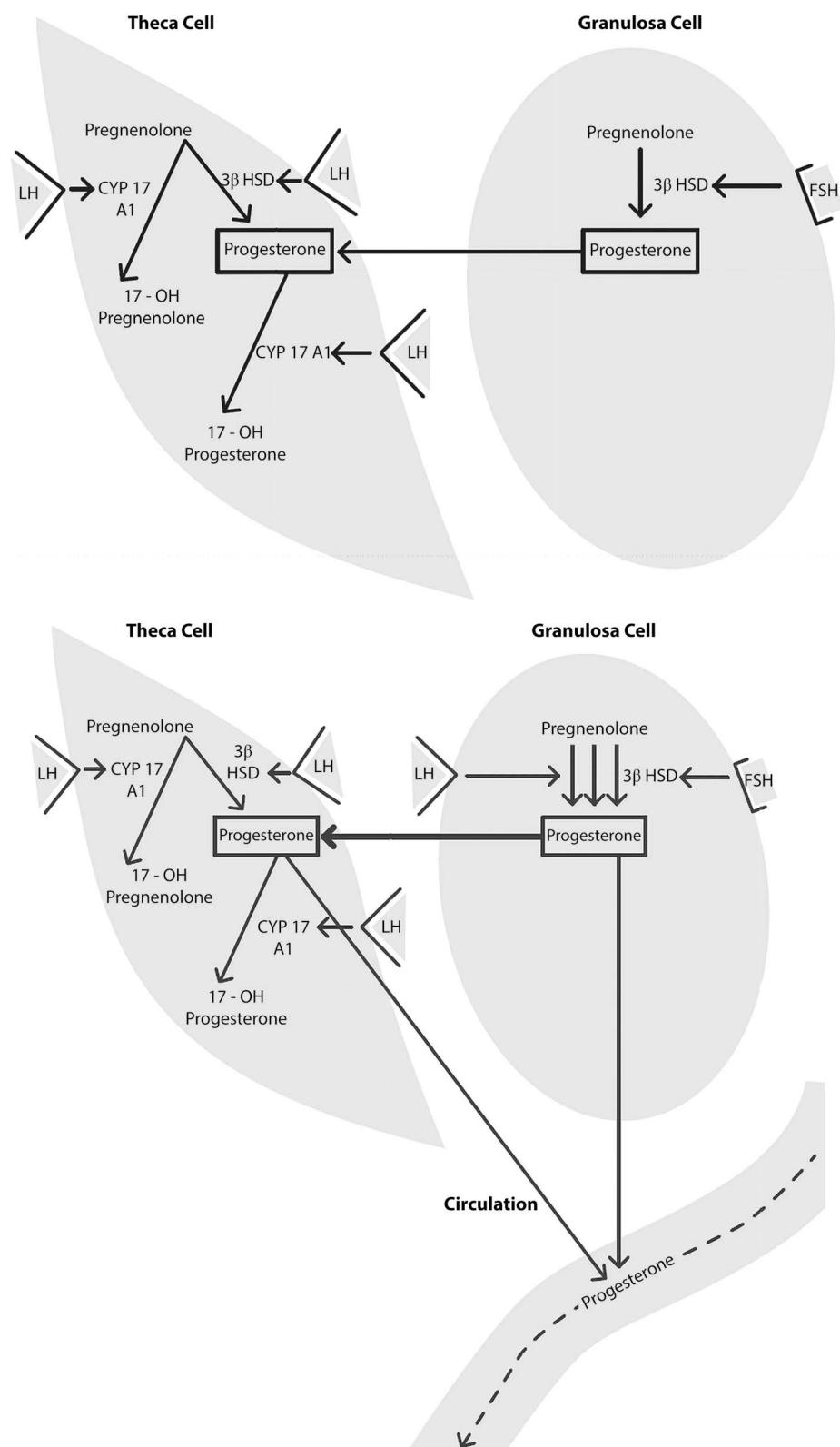


FIGURE 41.2 Progesterone synthesis and metabolism during the follicular phase. CYP 17 A1 (C17 hydroxylase) activity is only present in theca cells and hence progesterone produced by the granulosa cells diffuses into the theca cells to be hydroxylated. (a) During the early follicular phase, LH acts only on theca cells to stimulate 3 β HSD to convert pregnenolone to progesterone and CYP 17 A1 (C17 hydroxylase) to convert progesterone to 17-hydroxylated progesterone. (b) During the late follicular phase, LH receptors are heavily expressed on granulosa cells; the stimulatory effect of LH on progesterone production in the granulosa cells is three-fold stronger compared with FSH. Progesterone produced by granulosa cells may acquire access to the circulatory blood if produced in excess amounts.

The three major driving forces for LFPE are (i) the number of growing follicles, (ii) the dose of gonadotropins and their effect on the granulosa cells, and (iii), possibly, the effect of LH stimulation on the theca cells [32]. This is in line with reports of higher gonadotropin dose, oestradiol levels, and number of oocytes retrieved in patients with LFPE [27, 30, 33–35].

There is diurnal variation for serum progesterone in the late follicular phase, with higher values in the morning and lower values in the evening [36, 37]. The serum progesterone levels have been reported to decline significantly, by 44% between 8 and 20 hours on the day of triggering final oocyte maturation [36]. Such enormous variability in progesterone during the day suggests that a single progesterone determination on the final day of oocyte maturation may not be reliable enough to make clinical decisions.

The impact of late follicular phase progesterone elevation on reproductive outcome

The effect of LFPE on reproductive outcomes has been extensively studied. Although some studies have failed to demonstrate a detrimental effect of LFPE on reproductive outcomes [33, 38–40], the bulk of evidence shows a negative impact, both in the cleavage-stage and blastocyst-stage fresh embryo transfer cycles [30, 34, 41–45].

There is robust evidence that LFPE results in impaired endometrial receptivity. The pathophysiologic mechanisms for impaired endometrial receptivity include endometrial advancement, abnormal expression of implantation-regulating proteins, altered gene expression, or abnormal epigenetic profiles [46–49]. The deleterious effect of LFPE on endometrial receptivity is further supported by studies that failed to demonstrate any negative impact of LFPE during the OS cycle on the outcome of subsequent frozen embryo transfers [34, 35, 41, 50–52].

Another mechanism of action for a deleterious effect of LFPE on reproductive outcomes may be its effect of oocyte and embryo quality. A reduction in the number of top-quality embryos [42, 53], lower embryo utilization rates, and cumulative live birth rates have been reported in LFPE cycles [54, 55]. However, more recent evidence seems to refute such a detrimental effect; LFPE was neither associated with impaired embryonic development, increased rate of embryonic aneuploidy, nor compromised implantation and pregnancy outcomes following euploid frozen embryo transfer [56, 57]. Moreover, LFPE in the fresh cycle has been recently reported not to affect cumulative live birth rates when a freeze-all approach is adopted [58]. Given that endometrial receptivity in the presence of high progesterone is impaired [27, 44], fresh transfer of the *best* embryo of the cohort in patients with LFPE might be the main cause of the reported decreased cumulative pregnancy rates in earlier studies [55, 59]. The oocyte donation model is an excellent tool to assess the effects of LFPE on embryo quality independent of endometrial receptivity. The lack of a detrimental effect of LFPE on cumulative live birth rates in this model further adds evidence that LFPE does not affect embryo quality [60].

Finally, an intriguing feature seriously questioning the role of LFPE on oocyte/embryo quality is the effect of late-follicular phase intrafollicular progesterone concentration during a natural cycle. Schneyer et al. have reported that intrafollicular progesterone levels during the late-follicular phase in non-stimulated cycles may reach levels above 1700 ng/mL, which is more than a thousand times higher than the serum level cut-off of 1.5 ng/mL considered by most as clinically significant [61]. Despite the

lack of studies correlating intrafollicular and serum progesterone levels, the finding of such disparate levels raises a question about the existence of a pathophysiological mechanism by which LFPE could have any effect on oocyte and embryo quality.

Triggering of final oocyte maturation

Triggering ovulation is a crucial step in the management of OS in patients undergoing *in vitro* fertilization, intrauterine insemination, timed intercourse, and other forms of fertility therapy. Not only is the ovulation trigger responsible for the last stages of oocyte maturation and rupture of the follicle, but also it switches its granulosa cells to progesterone production, priming the endometrium for subsequent implantation. According to the current paradigm, this is caused by a critically timed, sustained elevation of oestradiol that culminates at the end of the follicular phase of the menstrual cycle.

Challenging this long-standing and universally accepted concept, a series of studies showed that in fact progesterone but not oestradiol may be the primary driver of ovulation in humans [62, 63]. During the follicular phase, progesterone levels are low and begin to rise even before the LH surge, and a prior study has shown that LH surge can be triggered by progesterone alone. By contrast, when progesterone activity remains continuously elevated above the gonadotropin surge-triggering threshold, as is the case with birth control formulations, during pregnancy, or the luteal phase, it probably causes desensitization of its own receptor or by proxy also GnRH receptors, so that LH surge is not possible, and ovulation is blocked [8, 63, 64].

LH is currently not preferred as an ovulation trigger in stimulated cycles. In ovarian stimulation cycles undertaken for IVF, due to its longer half-life and lower cost, hCG has been and is still used as an LH surrogate to trigger ovulation. Furthermore, hCG is the only agent that is approved by the FDA for this purpose. LH and hCG are characterized by specific molecular and biochemical features. They interact with distinct binding sites on the same receptor; however, the dissociation rates from these sites are lower for hCG compared with LH. Recombinant human LH has a shorter terminal half-life (around 10 hours) compared to hCG (terminal half-life 28 to 31 hours) [65]. The use of recombinant hCG and urinary hCG demonstrated the same efficacy for triggering final oocyte maturation during controlled ovarian stimulation protocols and represents the gold standard in fresh cycles [66].

The hCG bolus induces oocyte maturation, follicular luteinization, and stimulates endogenous progesterone production that is crucial for implantation. The luteal phase support provided by the traditional hCG bolus might not result in optimal progesterone concentrations in the early luteal phase and may possibly decrease implantation rates. The hCG bolus exerts a potentially premature and massive stimulation of the corpora lutea in the early luteal phase. This early stimulation of numerous corpora lutea often results in supra-physiological levels of progesterone in the early luteal phase, with progesterone levels reaching maximal levels about three days after oocyte collection. This contrasts with the natural cycle in which progesterone levels peak around the time of implantation in the midluteal phase.

GnRH agonists may be used to trigger final oocyte maturation in patients stimulated with a GnRH antagonist or PPOS protocol. This approach has been found to be particularly useful in women who are under risk for OHSS. Avoidance of hCG is desirable in these women, as prolonged exposure of the granulosa cells

from multiple growing follicles puts them under risk for OHSS. A GnRH agonist trigger is recommended for final oocyte maturation in women at risk of OHSS [67]. A Cochrane review of 17 RCTs ($n = 1847$) in a general patient population (i.e. including studies of women with both low and high risk of OHSS) reported a lower incidence of mild, moderate, or severe OHSS with a GnRH agonist trigger compared with an hCG trigger in autologous cycles (OR 0.15 [95% CI 0.05, 0.47], 8 RCTs, 989 women, IO = 42%, moderate-quality evidence) and donor-recipient cycles (OR 0.05 [95% CI 0.01, 0.28]; 3 RCTs, 374 women, IO = 0%) [68].

The GnRH agonist-induced surge more closely resembles the natural mid-cycle surge of gonadotropins, and exposes follicles to both LH and FSH. However, the surge lasts shorter and is much more attenuated after the GnRHa trigger, resulting in a poor support of corpora lutea that may lead to luteal phase deficiency. This necessitates modified/extensive luteal phase support if fresh transfer is contemplated.

Compared with hCG trigger, due to an additional FSH surge and the different effects of LH and hCG on the downstream signalling, the combined administration of hCG and GnRH agonist (dual/double trigger) for final oocyte maturation may be associated with significantly better cycle outcomes as shown in a recent meta-analysis [69]. In the included four studies of 527 women undergoing IVF treatment, women receiving a dual trigger had a significantly higher pregnancy rate compared with those receiving hCG alone (RR 1.55 [95% CI 1.17, 2.06]) [70]. A recently published RCT, randomizing 155 normal responder patients to receive either hCG or a dual trigger for final oocyte maturation, demonstrated that using a dual trigger for final follicular maturation resulted in improved outcomes [69]. Dual (administered at the same time), or double trigger (administration of hCG and GnRHa six hours apart), is an interesting option that needs to be pursued further; however, additional evidence must be provided to recommend this approach for all patients. Dual trigger may be further investigated in patients who have a discordance in the

number of oocytes/MII oocytes and MII oocytes/fertilized MII oocytes.

Several derivatives of kisspeptin are in the process of investigation for triggering ovulation. However, they are cumbersome to use, necessitating several injections or a pump, and are projected to be quite expensive when and if they reach the market [71]. The important shortcoming of all currently available ovulation trigger agents, including kisspeptin, is their inability to fully reproduce the naturally occurring pulsating pattern of GnRH release, which is believed to be a consequential feature of the process. This explains the deficient luteal phase and the need to support it with progesterone.

Luteal phase following ovarian stimulation for ART

Progesterone, the main product of corpus luteum, is indispensable for successful implantation and maintenance of early pregnancy. LH concentrations during the luteal phase of a spontaneous cycle range between 4–10 IU/L. This range of LH concentration suffices to produce a mid-luteal peak of progesterone production, which coincides with the time of implantation. A circulating mid-luteal progesterone level ≈ 10 ng/mL is generally considered to reflect ovulation and a normally functioning corpus luteum in a spontaneous cycle [72].

Luteal phase in stimulated ART cycles is defective [73–75]. Both the profile and duration of endogenous progesterone production in ART cycles are different as compared with the natural cycle. Firstly, the profile is different; following hCG trigger, there is a boost of progesterone production from multiple corpora lutea in the early luteal phase attaining peak levels exceeding 50 ng/mL three days after oocyte pick-up (OPU) (Figure 41.3) [76]. This is clearly different than the natural cycle, in which serum progesterone levels peak (≈ 10 ng/mL) in the mid-luteal phase coinciding

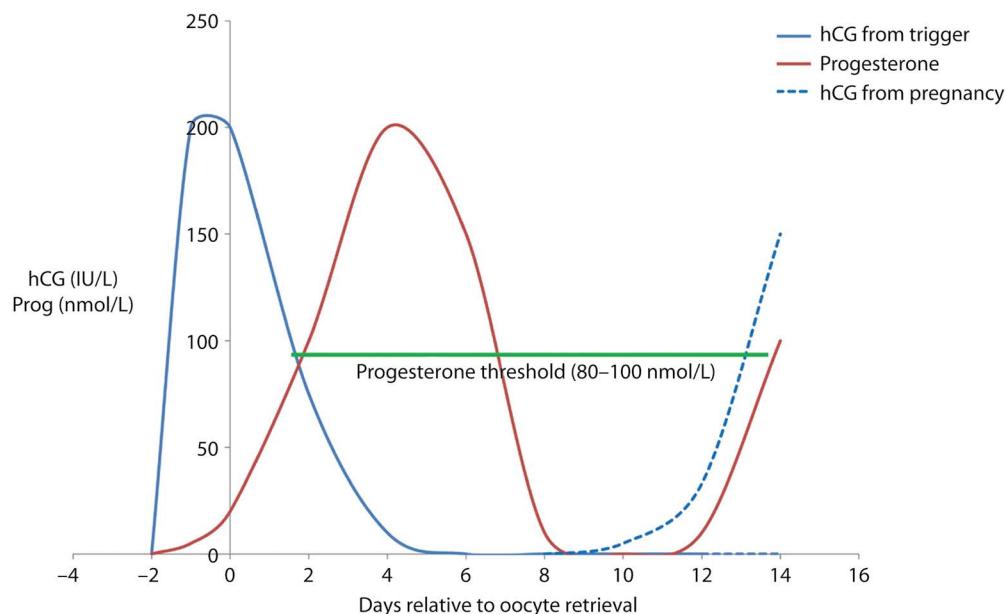


FIGURE 41.3 Circulating hCG and progesterone levels from hCG administration until early pregnancy during an *in vitro* fertilization cycle (1 nmol/L progesterone equals 0.31 ng/mL). Abbreviation: hCG, human chorionic gonadotropin.

with the window of implantation. Exposure to supra-physiological progesterone early in the luteal phase can cause endometrial advancement and impair endometrial receptivity. Secondly, the luteal phase lasts shorter in non-supplemented ART cycles due to premature luteolysis secondary to circulating supra-physiologic oestrogen and progesterone levels inhibiting endogenous LH secretion (Figure 41.3) [77]. Circulating mid-luteal LH levels are exceedingly low, being ≈ 0.2 IU/L after hCG and ≈ 1.5 IU/L after gonadotropin-releasing hormone agonist (GnRHa) triggering for final oocyte maturation [78]. Although the use of a bolus dose of hCG to trigger final oocyte maturation sparks progesterone production by the corpora lutea during the early luteal phase until the disappearance of hCG at 6–7 days following OPU [76, 79], there is a decline in progesterone production at the time of implantation due to the gradual decline in circulating hCG [80]. Hence, LPS is administered to compensate this gap when fresh embryo transfer is attempted [73, 81].

In theory, the circulating progesterone, although not ideal and affected by the route of exogenous progesterone administration, is still the best proxy for the endometrial progesterone concentration. However, in a recent study, following endometrial preparation using the hormone replacement therapy (HRT) protocol, serum progesterone levels were neither correlated with endometrial progesterone levels nor with endometrial receptivity as determined by endometrial receptivity assay (ERA) [82]. Endometrial progesterone and 17α -hydroxyprogesterone levels were positively correlated and related to endometrial receptivity by ERA. However, limited sample size and significant association of endometrial progesterone concentrations with ERA testing only for extreme endometrial progesterone levels as defined by relative maximum (quartile three + $1.5 \times$ interquartile distance) are the limitations of this study. In contrast, in an earlier study, patterns of endometrial gene expression have been reported to be dependent on concentrations of serum progesterone [83]. Moreover, measuring endometrial progesterone is not feasible and practical in daily clinical life.

Although daytime variations in serum progesterone during the mid-luteal phase in women undergoing IVF exist, a single progesterone measurement deemed to be low was reported to reflect the corpus luteum function quite accurately and, thus, detect patients with low circulating progesterone levels and subsequently suboptimal endometrial progesterone exposure [84].

During the luteal phase of an IVF and fresh embryo transfer cycle, progesterone production from multiple corpora lutea and exogenous administration of progesterone are the two sources contributing to the circulating progesterone. In the mid-luteal phase, the exogenously administered progesterone is responsible for $\approx 10\text{--}20\%$ of circulating progesterone levels, whereas the remaining $\approx 80\text{--}90\%$ is of ovarian origin [85]. Despite the use of some medications for triggering and LPS, there is significant interpersonal variation for both sources [84, 86–89].

In a recent study, Vuong et al. showed marked interpersonal variation in early luteal circulating progesterone levels following the same hCG trigger dose. Since a freeze-all policy was adopted in this study, the interpersonal variation during the early luteal phase was entirely due to differences in endogenous progesterone production from the corpora lutea [88]. These variations in endogenous production might, in theory, originate from differences in either responsiveness of corpora lutea (“quality” of corpora lutea) [84] and/or differences in serum concentrations of hCG during the early luteal phase used for triggering [90]. Both mechanisms of action may play a role. Not all corpora lutea are

of the same “quality”; this applies not only for a corpus luteum in a natural cycle [72] but also for multiple corpora lutea following ovarian stimulation [84].

In the study by Hull et al. [72] that analysed 212 cycles in 113 infertile women, the mean mid-luteal serum progesterone in the 21 untreated conception cycles was 12.8 ng/mL (95% CI: 8.8 to 16.7 ng/mL). The lowest serum progesterone value in a conception cycle was 8.5 ng/mL. Of note, neither the degree of follicular development (follicle diameter, oestradiol) nor the magnitude of LH surge (area under the curve-LH surge) determines the quantity of progesterone secreted after ovulation in a spontaneous cycle [91]. Thus, predicting patients with insufficient luteal progesterone levels is troublesome based on the follicular development, as abnormal luteal phases can be seen in cycles characterized by normal folliculogenesis [92].

As is the case in a natural cycle, the “quality” of multiple corpora lutea following OS might not necessarily be the same in terms of progesterone production. In a very important study, a possible daytime variation in progesterone secretion during the mid-luteal phase (OPU + seven days) was explored in 10 women undergoing IVF treatment [84]. The two patients with the lowest progesterone levels (11.3 and 17.3 ng/mL) had 17 and 19 follicles, respectively, on the day of OPU, showing that a large number of corpora lutea do not warrant a high progesterone output in the luteal phase. For this reason, monitoring of serum luteal phase progesterone could be of value to detect patients with low progesterone levels. Of note, there is no diurnal rhythm for serum progesterone in the mid-luteal phase in an IVF cycle [84]; hence, the accuracy of progesterone measurement is not improved by a fixed timing of blood sampling and could be performed at any time during clinic opening hours.

Hormone replacement therapy (HRT) for frozen/thawed embryo transfer is an ideal model to investigate the interpersonal differences in circulating progesterone levels following a standard dose of exogenous progesterone administration. Interestingly, marked interpersonal differences in circulating progesterone levels during the mid-luteal phase have been reported in HRT cycles despite the use of the same dose and route of progesterone administration [86]. Since there is no corpus luteum in such cycles, the marked interpersonal difference might be caused by altered pharmacokinetics of the administered progesterone, affected by body mass index (BMI) [93, 94] and female age [94, 95], as well as other intrinsic factors related to the patient [94]. Collectively, originating from these sources, there might be marked interpersonal differences in circulating luteal progesterone levels in fresh IVF transfer cycles.

Of interest, there is paucity of data regarding the impact of early luteal progesterone levels on the reproductive outcome of fresh embryo transfer cycles [87, 89, 96–100]. Two of these studies compared mid-luteal progesterone levels in pregnant versus non-pregnant patients [98, 99] and reported no difference in luteal progesterone levels between the two groups [98, 99]. Serial progesterone measurements in the early- and mid-luteal phase have also been reported in cleavage-stage fresh embryo transfer cycles with no difference in the profile between conception and non-conception cycles [96, 97, 100]. However, the majority of these studies [96–98] were published several decades ago and the protocols used for OS, LPS, and embryo transfer policy (number of embryos transferred and day of embryo transfer) do not match those that are currently used. Blastocyst-stage embryo transfer was employed in only one study [99]; however, in this study, serial progesterone measurement was not available and the circulating

progesterone levels on the day of embryo transfer were comparable among patients with and without live birth [99].

More recently, Thomsen et al. reported a non-linear relationship between circulating progesterone levels in the early (OPU +2/+3 day) and mid-luteal (OPU +5 day) phases [87]; low as well as high serum progesterone levels in the early and mid-luteal phases were associated with lower live birth rates following IVF with fresh embryo transfer. In this study, day 2/3/5 embryo transfers were performed and the lack of serial luteal progesterone measurements for all patients is a limitation; patients with cleavage-stage transfer ($n = 389$) had early luteal progesterone measurement available, whereas those with blastocyst-stage transfer ($n = 159$) had mid-luteal progesterone measurements. Of note, 11% and 51% of patients had suboptimal circulating progesterone levels during the early and mid-luteal phases, respectively. In contrast to this study, a more recent study employing day 2/3 embryo transfer and dydrogesterone-only for LPS failed to confirm the impact of an optimal window for progesterone during the early luteal phase [89].

Pulsatile secretion of progesterone in the mid-luteal phase is a limitation for luteal progesterone monitoring [84]. However, in the early luteal phase (OPU + 2 days), progesterone levels exhibit a non-pulsatile pattern both in natural and stimulated cycles [101, 102]. The magnitude of progesterone peaks in the mid-luteal phase (OPU + 7), however, has been reported to be significantly correlated to the median progesterone levels. Very large progesterone fluctuations were predominantly seen in patients with a mid-luteal progesterone concentration exceeding 78.6 ng/mL, whereas patients with progesterone levels <18.9 ng/mL displayed clinically stable progesterone values throughout the day [84]. Therefore, a patient with progesterone levels in the lower strata will be in the lower range despite pulsatile fluctuations. Further large-scale observational studies analysing serial serum progesterone assessment in patients undergoing fresh blastocyst transfer are clearly warranted to delineate the impact of the “quality” of the luteal phase on reproductive outcome measures.

A natural question that should be addressed is how to manage patients with suboptimal serum progesterone levels in the early or mid-luteal phases. Cycle cancellation or a rescue attempt with additional exogenous progesterone using the same or a different route of administration are the two options to manage these patients. To our knowledge, there is only one study evaluating the efficacy of “additional LPS” in patients undergoing fresh embryo transfer with low serum progesterone levels in the mid-luteal phase [103]. In a retrospective study, 1401 women who underwent their first IVF attempt following ovarian stimulation with a GnRH agonist protocol were included. A standard LPS (90 mg vaginal gel) was commenced on the day of OPU, and two good quality fresh cleavage-stage embryos were transferred in all patients. Serum progesterone level was assessed six days after embryo transfer (nine days after OPU); patients with progesterone levels <10 ng/mL were administered oral dydrogesterone, 10 mg twice daily, in addition to standard LPS. Of note, such a rescue protocol attained live birth rates (~68%) that were comparable to those patients with mid-luteal serum progesterone >40 ng/mL. Obviously, further studies are warranted to explore the rescue protocols, in terms of timing, the route, and the dose of additional progesterone administration, in patients undergoing fresh embryo transfer with suboptimal early and mid-luteal serum progesterone levels.

Collectively, these data clearly indicate that luteal phase has been the most ignored segment of an IVF treatment cycle. There

is paucity of data examining the optimal luteal progesterone levels in fresh embryo transfer cycles and, therefore, a lack of defined progesterone thresholds and/or luteal progesterone profiles to be used for clinical decision-making. Hopefully, with the availability of such data, a personalized approach will be available, rather than the impersonalized, standard LPS without luteal progesterone monitoring, which is currently the “standard of care” and common practice in IVF programs following fresh embryo transfer.

Androgens

Androgens are produced by the theca cells and serve as a substrate for oestrogen biosynthesis. Androgen receptors (ARs) are expressed in the theca cells, granulosa cells, and ova [104]. Androgens may exert autocrine and paracrine effects in regulating follicular function [105]. Androgens also upregulate their receptors and augment FSH receptors on granulosa cells [106]. Studies in primates show that testosterone treatment increases FSH receptors in granulosa cells, stimulates early stages of follicle growth, and increases the numbers of preantral and antral follicles [107]. Similarly, there is a strong correlation between follicular fluid testosterone levels and FSH receptor expression in human granulosa cells from the small (3–9 mm) antral follicles [106]. The number of AR-positive follicles increases at each progressive growth stage, suggesting a role for androgens in promoting early follicle growth [108].

In premenopausal women, serum testosterone concentrations decrease with age [109]. This decline mirrors the decrease in anti-Mullerian hormone (AMH) levels and antral follicle counts. Ovarian testosterone increases the response of antral follicles to ovarian stimulation [110], mediated or potentiated by IGF-I. Although androgens synergistically act with FSH to support folliculogenesis, and ovarian androgen secretion declines with age, there is still no conclusive evidence that androgen therapy is effective in improving ovarian FSH sensitivity [111].

Increased circulating levels of insulin and IGF-I and exogenous testosterone and increased local ovarian testosterone concentrations due to aromatase inhibition or exogenous LH/hCG are all associated with an increased ovarian response to gonadotropins. These theoretical possibilities led to treatment strategies aimed at increasing circulating or local androgens in poor responders.

HCG and rLH have long been used as adjuvant agents for increasing the production of endogenous intraovarian androgens through the addition of LH activity. There is some evidence that addition of LH activity to ovarian stimulation may benefit poor responder patients >35 years of age [22, 23].

Transdermal testosterone or dehydroepiandrosterone (DHEA) administered prior to OS have been suggested as safe and effective ways of increasing intraovarian androgen concentrations, thus increasing the sensitivity of the ovary to stimulation [112]. Recently published RCTs have evaluated transdermal testosterone [113, 114] or DHEA pre-treatment [115] in poor responders undergoing ovarian stimulation for IVF, however, with inconclusive results.

A more recent systematic review and network meta-analysis concluded that, among treatments aimed at replacing or increasing androgen concentrations, only DHEA supplementation might increase clinical pregnancy rates in women with a diminished ovarian reserve [116].

Absence of properly powered RCTs of androgen supplementation in women with low ovarian reserve is the main reason why

androgen pre-treatment or co-treatment has remained controversial. Although several RCTs have been published, none achieved adequate power and/or involved appropriately selected patient populations both in treatment and control groups [117]. Thus, call for proper RCTs instead of more meta-analyses on the subject is timely [118].

Unconventional ovarian stimulation

Classical dogma dictates the initiation of OS in the early follicular phase. The rationale is the simultaneous stimulation of a synchronous cohort of antral follicles recruited during the luteo-follicular transition. Interestingly, there is increasing evidence to indicate that multiple waves of antral follicles develop during one menstrual cycle, challenging the concept of a single recruitment episode during the follicular phase [119]. Approximately two-thirds of women develop two follicle waves throughout an interovulatory interval and the remainder exhibit three waves of follicle development. Major and minor waves of follicle development have been observed. Major waves are those in which a dominant follicle develops; dominant follicles either regress or ovulate. In minor waves, physiologic selection of a dominant follicle is not manifest. Knowledge of waves of antral follicular development has led to the global adoption of novel ovarian stimulation strategies in which stimulation can be initiated at various times throughout the cycle. Random-start and luteal-phase ovarian stimulation regimens have had important clinical applications for women requiring urgent oocyte or embryo cryopreservation for fertility preservation prior to chemotherapy [120].

Luteal-phase stimulation protocols appear to be as successful as follicular-phase stimulation protocols in terms of the number of collected oocytes, embryos formed, and clinical pregnancy rates following the transfer of thawed embryos [121]. Luteal-phase ovarian stimulation has also been explored in women with poor ovarian reserve with favourable results [122]. As the endometrium is out of phase following luteal-phase stimulation, embryo freezing followed by a frozen embryo transfer in a subsequent cycle is required [123, 124]. As for cancer patients, initiating emergency ovarian stimulation for fertility preservation in the follicular versus the luteal phase yielded similar numbers of oocytes and MII oocytes [125].

Performing follicular-phase stimulation and luteal-phase stimulation in the same menstrual cycle, named as double stimulation/dual stimulation, increases the number of oocytes, which appears to be a robust surrogate marker of live birth rate in IVF across all female ages [126]. Of interest, apart from one study, the bulk of evidence reports significantly higher numbers of oocytes following luteal-phase stimulation when compared with follicular-phase stimulation [127].

Some follicles recruited at the start of the luteo-follicular transition may have already reached the pre-ovulatory stage early in the follicular phase. Thus, it is also possible to collect mature oocytes early in the follicular phase that are capable of leading to a live birth [128].

It may be concluded that OS may be undertaken with unconventional means that challenge the current dogma of universal follicular-phase stimulation.

Conclusions

The endocrine profile of a stimulated ART cycle is different from that of the natural cycle during both the follicular and luteal

phases. Overshooting the FSH threshold is clearly mandatory for stimulation of multi-follicular growth. Serum FSH values are not useful to predict the extent of multi-follicular growth, thus routine monitoring of it can be unnecessary for most patients, but can provide critical information in some instances and requires further research. Despite the clear requirement for some LH activity for proper follicle growth, an LH threshold is not determined and evidence to support routine monitoring of serum LH levels during stimulation is missing. Serum oestradiol levels are higher in ART cycles than in the natural cycle and reflect the extent of multi-follicular growth. Even though different patterns of oestradiol during stimulation can be related to treatment outcome, e.g. predicting pregnancy or the occurrence of OHSS, currently available evidence does not demonstrate a clear advantage of monitoring serum oestradiol levels over ultrasound-only monitoring of the ART cycle [129]. If the ultrasound examination prior to commencement of gonadotropins fails to confirm pituitary suppression in the long luteal GnRH agonist protocol, serum levels of LH, oestradiol, and progesterone can be measured, together or separately, for confirmation. While the low incidence of progesterone elevation at the start of GnRH antagonist cycles precludes routine measurement of progesterone levels at this stage, elevated progesterone levels during the late follicular phase seem to have implications for treatment outcome and can thus be informative for clinical decision-making. Some experts advocate monitoring luteal-phase serum progesterone levels for tailoring luteal support protocols in the presence of low progesterone levels. However, more information is required before implementing luteal phase progesterone monitoring into routine practice.

References

- Pache TD, Wladimiroff JW, de Jong FH, Hop WC, Fauser BC. Growth patterns of nondominant ovarian follicles during the normal menstrual cycle. *Fertil Steril*. 1990;54:638–42.
- Polyzos NP, Drakopoulos P, Parra J, et al. Cumulative live birth rates according to the number of oocytes retrieved after the first ovarian stimulation for in vitro fertilization/intracytoplasmic sperm injection: A multicenter multinational analysis including ~15,000 women. *Fertil Steril* 2018;110(4):661–70.
- Dahan MH, Tannus S, Seyhan A, Tan SL, Ata B. Combined modalities for the prevention of ovarian hyperstimulation syndrome following an excessive response to stimulation. *Gynecol Endocrinol*. 2018;34(3):252–5.
- van Weissenbruch MM, Schoemaker HC, Drexhage HA, Schoemaker J, Pharmaco-dynamics of human meno-pausal gonadotrophin (HMG) and follicle-stimulating hormone (FSH). The importance of the FSH concentration in initiating follicular growth in polycystic ovary-like disease. *Hum Reprod*. 1993;8:813–2.
- Lensen SF, Wilkinson J, Leijdekkers JA, et al. Individualised gonadotropin dose selection using markers of ovarian reserve for women undergoing in vitro fertilisation plus intracytoplasmic sperm injection (IVF/ICSI). *Cochrane Database Syst Rev*. 2018;2(2):CD012693.
- Fatemi H, Bilger W, Denis D, et al. Dose adjustment of follicle-stimulating hormone (FSH) during ovarian stimulation as part of medically-assisted reproduction in clinical studies: A systematic review covering 10 years (2007–2017). *Reprod Biol Endocrinol*. 2021;19(1):68.
- Ebid AHIM, Abdel Motaleb SM, Mostafa MI, Soliman MMA. Population PK-PD-PD modeling of recombinant follicle stimulating hormone in in vitro Fertilization/Intracytoplasmic sperm injection: Implications on dosing and timing of gonadotrophin therapy. *J Clin Pharmacol*. 2021;61(5):700–13.

8. Ata B, Capuzzo M, Turkgeldi E, Yildiz S, La Marca A. Progestins for pituitary suppression during ovarian stimulation for ART: A comprehensive and systematic review including meta-analyses. *Hum Reprod Update*. 2021;27(1):48–66.
9. La Marca A, Capuzzo M. Use of progestins to inhibit spontaneous ovulation during ovarian stimulation: The beginning of a new era? *Reprod Biomed Online*. 2019;39(2):321–31.
10. Loumaye E, Engrand P, Shoham Z, Hillier SG, Baird DT. Clinical evidence for an LH “ceiling” effect induced by administration of recombinant human LH during the late follicular phase of stimulated cycles in world health organization type I and type II anovulation. *Hum Reprod*. 2003;18:314–22.
11. Balasch J, Fabregues F. Is luteinizing hormone needed for optimal ovulation induction? *Curr Opin Obstet Gynecol*. 2002;14:265–74.
12. Shoham Z. The clinical therapeutic window for luteinizing hormone in controlled ovarian stimulation. *Fertil Steril*. 2002;77:1170–7.
13. Hillier SG. Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Hum Reprod*. 1994;9:188–91.
14. Filicori M, Cognigni GE, Pocognoli P, Ciampaglia W, Bernardi S. Current concepts and novel applications of LH activity in ovarian stimulation. *Trends Endocrinol Metab*. 2003;14:267–73.
15. Filicori M, Cognigni GE, Tabarelli C, et al. Stimulation and growth of antral ovarian follicles by selective LH activity administration in women. *J Clin Endocrinol Metab*. 2002;87:1156–61.
16. Griesinger G, Shapiro DB. Luteinizing hormone add-back: Is it needed in controlled ovarian stimulation, and if so, when? *J Reprod Med*. 2011;56:279–300.
17. Griesinger G, Shapiro DB, Kolibianakis EM, Witjes H, Mannaerts BM. No association between endogenous LH and pregnancy in a GnRH antagonist protocol: Part II, recombinant FSH. *Reprod Biomed Online*. 2011;23:457–65.
18. van Wely M, Kwan I, Burt AL, et al. Recombinant versus urinary gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. A Cochrane Review. *Hum Reprod Update*. 2012;18:111.
19. van Wely M, Kwan I, Burt AL, et al. Recombinant versus urinary gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. *Cochrane Database Syst Rev*. 2011;2:CD005354.
20. Al-Inany HG, van Gelder P. Effect of urinary versus recombinant FSH on clinical outcomes after frozen-thawed embryo transfers: A systematic review. *Reprod Biomed Online*. 2010;21:151–8.
21. Requena A, Cruz M, Ruiz FJ, Garcia-Velasco JA. Endocrine profile following stimulation with recombinant follicle stimulating hormone and luteinizing hormone versus highly purified human menopausal gonadotropin. *Reprod Biol Endocrinol*. 2014;12:10.
22. Conforti A, Esteves SC, Humaidan P, et al. Recombinant human luteinizing hormone co-treatment in ovarian stimulation for assisted reproductive technology in women of advanced reproductive age: A systematic review and meta-analysis of randomized controlled trials. *Reprod Biol Endocrinol*. 2021;19(1):91.
23. Alviggi C, Conforti A, Esteves SC, et al. International Collaborative Group for the Study of r-hLH (iCOS-LH). Recombinant luteinizing hormone supplementation in assisted reproductive technology: A systematic review. *Fertil Steril*. 2018;109(4):644–64.
24. Hamdine O, Macklon NS, Eijkemans MJ, et al. Elevated early follicular progesterone levels and *in vitro* fertilization outcomes: A prospective intervention study and meta-analysis. *Fertil Steril*. 2014;102:448–54.
25. Kolibianakis EM, Zikopoulos K, Smitz J, et al. Elevated progesterone at initiation of stimulation is associated with a lower ongoing pregnancy rate after IVF using GnRH antagonists. *Hum Reprod*. 2004;19:1525–9.
26. Blockeel C, Baumgarten M, De Vos M, Verheyen G, Devroey P. Administration of GnRH antagonists in case of elevated progesterone at initiation of the cycle: A prospective cohort study. *Curr Pharm Biotechnol*. 2011;12:423–8.
27. Venetis CA, Kolibianakis EM, Bosdou JK, Tarlatzis BC. Progesterone elevation and probability of pregnancy after IVF: A systematic review and meta-analysis of over 60 000 cycles. *Hum Reprod Update*. 2013;19:433–57.
28. De Geyter C, De Geyter M, Huber PR, Nieschlag E, Holzgreve W. Progesterone serum levels during the follicular phase of the menstrual cycle originate from the crosstalk between the ovaries and the adrenal cortex. *Hum Reprod*. 2002;17:933–9.
29. Keck C, Neulen J, Breig-Lauel S, Breckwoldt M. Elevated serum progesterone concentrations during the early follicular phase of the menstrual cycle: Clinical significance and therapeutic implications. *Gynecol Endocrinol*. 1999;13:161–5.
30. Bosch E, Labarta E, Crespo J, et al. Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for *in vitro* fertilization: Analysis of over 4000 cycles. *Hum Reprod*. 2010;25:2092–100.
31. Oktem O, Akin N, Bildik G, et al. FSH stimulation promotes progesterone synthesis and output from human granulosa cells without luteinization. *Hum Reprod*. 2017;32:643–52.
32. Fleming R, Jenkins J. The source and implications of progesterone rise during the follicular phase of assisted reproduction cycles. *Reprod Biomed Online*. 2010;21(4):446–9.
33. Martinez F, Rodriguez I, Devesa M, Buxaderas R, Gómez MJ, Coroleu B. Should progesterone on the human chorionic gonadotropin day still be measured? *Fertil Steril*. 2016;105(1):86–92.
34. Xu B, Li Z, Zhang H, et al. Serum progesterone level effects on the outcome of in vitro fertilization in patients with different ovarian response: An Analysis of more than 10,000 cycles. *Fertil Steril*. 2012;97(6):1321–7.e1–4.
35. Healy MW, Yamasaki M, Patounakis G, et al. The slow growing embryo and premature progesterone elevation: Compounding factors for embryo-endometrial asynchrony. *Hum Reprod*. 2017;32:362–67.
36. González-Foruria I, Rodríguez I, Martínez F, et al. Clinically significant intra-day variability of serum progesterone levels during the final day of oocyte maturation: A prospective study with repeated measurements. *Hum Reprod*. 2019;34:1551–8.
37. Shanker U, Lawrenz B, Bungum L, et al. Significant serum progesterone variations on the day of final oocyte maturation in stimulated IVF cycles. *Front Endocrinol*. 2019;10:806.
38. Griesinger G, Mannaerts B, Andersen CY, Witjes H, Kolibianakis EM, Gordon K. Progesterone elevation does not compromise pregnancy rates in high responders: A pooled analysis of *in vitro* fertilization patients treated with recombinant follicle-stimulating hormone/gonadotropin-releasing hormone antagonist in six trials. *Fertil Steril*. 2013;100:1622–8.e1–3.
39. Martínez F, Coroleu B, Clua E, et al. Serum progesterone concentrations on the day of HCG administration cannot predict pregnancy in assisted reproduction cycles. *Reprod Biomed Online*. 2004;8:183–90.
40. Requena A, Cruz M, Bosch E, Meseguer M, García-Velasco JA. High progesterone levels in women with high ovarian response do not affect clinical outcomes: A retrospective cohort study. *Reprod Biol Endocrinol*. 2014;12:69.
41. Huang R, Fang C, Xu S, Yi Y, Liang X. Premature progesterone rise negatively correlated with live birth rate in IVF cycles with GnRH agonist: An Analysis of 2,566 cycles. *Fertil Steril*. 2012;98:664–70.e662.
42. Huang B, Ren X, Wu L, et al. Elevated progesterone levels on the day of oocyte maturation May affect top quality embryo IVF cycles. *PLoS One*. 2016;11:e0145895.
43. Santos-Ribeiro S, Polyzos NP, Haentjens P, et al. Live birth rates after IVF are reduced by both low and high progesterone levels on the day of human chorionic gonadotrophin administration. *Hum Reprod*. 2014;29:1698–705.
44. Venetis CA, Kolibianakis EM, Bosdou JK, et al. Estimating the net effect of progesterone elevation on the day of hCG on live birth rates after IVF: A cohort analysis of 3296 IVF cycles. *Hum Reprod*. 2015;30:684–691.

45. Bozdag G, Turkyilmaz E, Yildiz S, Mumusoglu S, Yarali H. Progesterone elevation and preventive strategies to avoid implantation failure. *Semin Reprod Med.* 2019;37:265–72.
46. Horcajadas JA, Riesewijk A, Polman J, et al. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. *Mol Hum Reprod.* 2005;11:195–205.
47. Labarta E, Martínez-Conejero JA, Alamá P, et al. Endometrial receptivity is affected in women with high circulating progesterone levels at the end of the follicular phase: A functional genomics analysis. *Hum Reprod.* 2011;26:1813–25.
48. Van Vaerenbergh I, Fatemi HM, Blockeel C, et al. Progesterone rise on HCG day in GnRH antagonist/rFSH stimulated cycles affects endometrial gene expression. *Reprod Biomed Online.* 2011;22:263–71.
49. Xiong Y, Wang J, Liu L, et al. Effects of high progesterone level on the day of human chorionic gonadotrophin administration in *in vitro* fertilization cycles on epigenetic modification of endometrium in the peri-implantation period. *Fertil Steril.* 2017;108:269–76.e261.
50. Baldini D, Savoia MV, Sciancalepore AG, et al. High progesterone levels on the day of HCG administration do not affect the embryo quality and the reproductive outcomes of frozen embryo transfers. *Clin Ter.* 2018;169:e91–5.
51. Healy MW, Patounakis G, Connell MT, et al. Does a frozen embryo transfer ameliorate the effect of elevated progesterone seen in fresh transfer cycles? *Fertil Steril.* 2016;105:93–99.e91.
52. Yang S, Pang T, Li R, et al. The individualized choice of embryo transfer timing for patients with elevated serum progesterone level on the HCG day in IVF/ICSI cycles: A prospective randomized clinical study. *Gynecol Endocrinol.* 2015;31:355–8.
53. Vanni VS, Somigliana E, Reschini M, et al. Top quality blastocyst formation rates in relation to progesterone levels on the day of oocyte maturation in GnRH antagonist IVF/ICSI cycles. *PLoS One.* 2017;12:e0176482.
54. Bu Z, Zhao F, Wang K, et al. Serum progesterone elevation adversely affects cumulative live birth rate in different ovarian responders during *in vitro* fertilization and embryo transfer: A large retrospective study. *PLoS One.* 2014;9:e100011.
55. Racca A, Santos-Ribeiro S, De Munck N, et al. Impact of late-follicular phase elevated serum progesterone on cumulative live birth rates: Is there a deleterious effect on embryo quality? *Hum Reprod.* 2018;33:860–68.
56. Hernandez-Nieto C, Lee JA, Alkon-Meadows T, et al. Late follicular phase progesterone elevation during ovarian stimulation is not associated with decreased implantation of chromosomally screened embryos in thaw cycles. *Hum Reprod.* 2020;35:1889–99.
57. Neves AR, Santos-Ribeiro S, García-Martínez S, et al. The effect of late-follicular phase progesterone elevation on embryo ploidy and cumulative live birth rates. *Reprod Biomed Online.* 2021;43:1063–9.
58. Racca A, Vanni VS, Somigliana E, et al. Is a freeze-all policy the optimal solution to circumvent the effect of late follicular elevated progesterone? A multicentric matched-control retrospective study analysing cumulative live birth rate in 942 non-elective freeze-all cycles. *Hum Reprod.* 2021;36:2463–72.
59. Vanni VS, Alleva E, Papaleo E, Candiani M, Somigliana E, Viganò P. Assessing The clinical significance of elevated progesterone during controlled ovarian stimulation: The unanswered question about embryo quality. *Hum Reprod.* 2018;33:1191–2.
60. Racca A, De Munck N, Santos-Ribeiro S, et al. Do we need to measure progesterone in oocyte donation cycles? A retrospective analysis evaluating cumulative live birth rates and embryo quality. *Hum Reprod.* 2020;35:167–74.
61. Schneyer AL, Fujiwara T, Fox J, et al. Dynamic changes in the intrafollicular inhibin/activin/follistatin axis during human follicular development: Relationship to circulating hormone concentrations. *J Clin Endocrinol Metab.* 2000;85:3319–30.
62. Zalanyi S. Progesterone and ovulation. *Eur J Obstet Gynecol Reprod Biol.* 2001;98(2):152–9.
63. Buchholz R, Nocke I, Nocke W. The influence of gestagens on the urinary excretion of pituitary gonadotropins, estrogens, and pregnanediol in women in the postmenopause and during the menstrual cycle. *Int J Fertil.* 1964;9:231–51.
64. Dozortsev DI, Pellicer A, Diamond MP. Progesterone is a physiological trigger of ovulatory gonadotropins. *Fertil Steril.* 2020;113(5):923–4.
65. Troppmann B, Kleinau G, Krause G, Gromoll J. Structural and functional plasticity of the luteinizing hormone/choriogonadotrophin receptor. *Hum Reprod Update.* 2013;19(5):583–602.
66. Youssef MA, Abou-Setta AM, Lam WS. Recombinant versus urinary human chorionic gonadotrophin for final oocyte maturation triggering in IVF and ICSI cycles. *Cochrane Database Syst Rev.* 2016;4: CD003719.
67. Orvieto R, Venetis CA, Fatemi HM, et al. Optimising follicular development, pituitary suppression, triggering and luteal phase support during assisted reproductive technology: A delphi consensus. *Front Endocrinol.* 2021;12:675670.
68. Youssef MA, Van der Veen F, Al-Inany HG, et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database Syst Rev.* 2014;10:CD008046.
69. Haas J, Bassil R, Samara N, et al. GnRH agonist and hCG (dual trigger) versus hCG trigger for final follicular maturation: A double-blinded, randomized controlled study. *Hum Reprod.* 2020;35(7):1648–54.
70. Ding N, Liu X, Jian Q, et al. Dual trigger of final oocyte maturation with a combination of GnRH agonist and hCG versus a hCG alone trigger in GnRH antagonist cycle for *in vitro* fertilization: A systematic review and meta-analysis. *Eur J Obstet Gynecol Reprod Biol.* 2017;218:92–8.
71. Abbara A, Clarke S, Islam R, et al. A second dose of kisspeptin-54 improves oocyte maturation in women at high risk of ovarian hyperstimulation syndrome: A phase 2 randomized controlled trial. *Hum Reprod.* 2017;32(9):1915–24.
72. Hull MG, Savage PE, Bromham DR, Ismail AA, Morris AF. The value of a single serum progesterone measurement in the midluteal phase as a criterion of a potentially fertile cycle (“ovulation”) derived from treated and untreated conception cycles. *Fertil Steril.* 1982;37:355–60.
73. Edwards RG, Steptoe PC, Purdy JM. Establishing full-term human pregnancies using cleaving embryos grown *in vitro*. *Br J Obstet Gynaecol.* 1980;87:737–56.
74. Jones HW Jr. What has happened? Where are we? *Hum Reprod.* 1996;11(Suppl 1):7–24; discussion 29–31.
75. Fatemi HM, Popovic-Todorovic B, Papanikolaou E, Donoso P, Devroey P. An update of luteal phase support in stimulated IVF cycles. *Hum Reprod Update.* 2007;13:581–90.
76. Fauser BC, de Jong D, Olivennes F, et al. Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for *in vitro* fertilization. *J Clin Endocrinol Metab.* 2002;87:709–15.
77. Beckers NG, Macklon NS, Eijkemans MJ, et al. Non-supplemented luteal phase characteristics after the administration of recombinant human chorionic gonadotropin, recombinant luteinizing hormone, or gonadotropin-releasing hormone (GnRH) agonist to induce final oocyte maturation in *in vitro* fertilization patients after ovarian stimulation with recombinant follicle-stimulating hormone and GnRH antagonist cotreatment. *J Clin Endocrinol Metab.* 2003;88:4186–92.
78. Humaidan P, Bredkjaer HE, Bungum L, et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomized study. *Hum Reprod.* 2005;20:1213–20.

79. Weissman A, Lurie S, Zalel Y, Goldchmit R, Shoham Z. Human chorionic gonadotropin: Pharmacokinetics of subcutaneous administration. *Gynecol Endocrinol.* 1996;10:273–6.
80. Yding Andersen C, Vilbour Andersen K. Improving the luteal phase after ovarian stimulation: Reviewing new options. *Reprod Biomed Online.* 2014;28(5):552–9.
81. van der Linden M, Buckingham K, Farquhar C, Kremer JA, Metwally M. Luteal phase support for assisted reproduction cycles. *Cochrane Database Syst Rev.* 2015;2015(7):CD009154.
82. Labarta E, Sebastian-Leon P, Devesa-Peiro A, et al. Analysis of serum and endometrial progesterone in determining endometrial receptivity. *Hum Reprod.* 2021;36:2861–70.
83. Young SL, Savaris RF, Lessey BA, et al. Effect of randomized serum progesterone concentration on secretory endometrial histologic development and gene expression. *Hum Reprod.* 2017;32:1903–14.
84. Thomsen LH, Kesmodel US, Andersen CY, Humaidan P. Daytime variation in serum progesterone during the mid-luteal phase in women undergoing in vitro fertilization treatment. *Front Endocrinol.* 2018;9:92.
85. Kol S, Homburg R. Change, change, change: Hormonal actions depend on changes in blood levels. *Hum Reprod.* 2008;23:1004–6.
86. Mumusoglu S, Polat M, Ozbek IY, et al. Preparation of the endometrium for frozen embryo transfer: A systematic review. *Front Endocrinol.* 2021;12:688237.
87. Thomsen LH, Kesmodel US, Erb K, et al. The impact of luteal serum progesterone levels on live birth rates—a prospective study of 602 IVF/ICSI cycles. *Hum Reprod.* 2018;33:1506–16.
88. Vuong LN, Ho TM, Pham TD, Ho VNA, Andersen CY, Humaidan P. The early luteal hormonal profile in IVF patients triggered with hCG. *Hum Reprod.* 2020;35:157–66.
89. Netter A, Mancini J, Buffat C, Agostini A, Perrin J, Courbiere B. Do early luteal serum progesterone levels predict the reproductive outcomes in IVF with oral dydrogesterone for luteal phase support? *PLoS One.* 2019;14:e0220450.
90. Vuong LN, Pham TD, Ho VNA, Ho TM, Humaidan P, Andersen CY. Determinants of the hCG concentration in the early luteal phase after final maturation of follicles with bolus trigger of recombinant hCG. *Front Endocrinol.* 2020;11:137.
91. Soules MR, Clifton DK, Steiner RA, Cohen NL, Bremner WJ. The corpus luteum: Determinants of progesterone secretion in the normal menstrual cycle. *Obstet Gynecol.* 1988;71:659–66.
92. Grunfeld L, Sandler B, Fox J, Boyd C, Kaplan P, Navot D. Luteal phase deficiency after completely normal follicular and periovulatory phases. *Fertil Steril.* 1989;52:919–23.
93. Brady PC, Kaser DJ, Ginsburg ES, et al. Serum progesterone concentration on day of embryo transfer in donor oocyte cycles. *J Assist Reprod Genet.* 2014;31:569–75.
94. González-Foruria I, Gaggiotti-Marre S, Álvarez M, et al. Factors associated with serum progesterone concentrations the day before cryopreserved embryo transfer in artificial cycles. *Reprod Biomed Online.* 2020;40(6):797–804.
95. Levy T, Yairi Y, Bar-Hava I, Shalev J, Orvieto R, Ben-Rafael Z. Pharmacokinetics of the progesterone-containing vaginal tablet and its use in assisted reproduction. *Steroids.* 2000;65:645–49.
96. Howles CM, Macnamee MC, Edwards RG. Follicular development and early luteal function of conception and non-conceptional cycles after human in-vitro fertilization: Endocrine correlates. *Hum Reprod.* 1987;2:17–21.
97. Hassiakos D, Toner JP, Muasher SJ, Jones HW Jr. Implantation and pregnancy rates in relation to oestradiol and progesterone profiles in cycles with and without the use of gonadotrophin-releasing hormone agonist suppression. *Hum Reprod.* 1990;5:1004–8.
98. Balasch J, Creus M, Fabregues F, et al. Hormonal profiles in successful and unsuccessful implantation in IVF-ET after combined GnRH agonist/gonadotropin treatment for superovulation and hCG luteal support. *Gynecol Endocrinol.* 1995;9:51–8.
99. Petersen JF, Andersen AN, Klein BM, Helmgård L, Arce JC. Luteal phase progesterone and oestradiol after ovarian stimulation: Relation to response and prediction of pregnancy. *Reprod Biomed Online.* 2018;36:427–34.
100. Sonntag B, Loebbecke KC, Nofer JR, Kiesel L, Greb RR. Serum estradiol and progesterone in the mid-luteal phase predict clinical pregnancy outcome in IVF/ICSI cycles. *Gynecol Endocrinol.* 2013;29:700–3.
101. Filicori M, Butler JP, Crowley WF Jr. Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion. *J Clin Invest.* 1984;73:1638–47.
102. Tannus S, Burke Y, McCartney CR, Kol S. GnRH-agonist triggering for final oocyte maturation in GnRH-antagonist IVF cycles induces decreased LH pulse rate and amplitude in early luteal phase: A possible luteolysis mechanism. *Gynecol Endocrinol.* 2017;33:741–5.
103. Tu J, Lin G, Gong F. Additional luteal support might improve IVF outcomes in patients with low progesterone level in middle luteal phase following a GnRH agonist protocol. *Gynecol Endocrinol.* 2020;37(2):1–5.
104. Astapova O, Minor BMN, Hammes SR. Physiological and pathological androgen actions in the ovary. *Endocrinology.* 2019;160:1166–74.
105. Suzuki T, Sasano H, Kimura N, et al. Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: Relationship to expression of steroidogenic enzymes. *Hum Reprod.* 1994; 9(9):1589–95.
106. Nielsen ME, Rasmussen IA, Kristensen SG, et al. In human granulosa cells from small antral follicles, androgen receptor mRNA and androgen levels in follicular fluid correlate with FSH receptor mRNA. *Mol Hum Reprod.* 2011;17(1):63–70.
107. Weil S, Vendola K, Zhou J, Bondy CA. Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. *J Clin Endocrinol Metab.* 1999;84(8):2951–56.
108. Rice S, Ojha K, Whitehead S, Mason H. Stage-specific expression of androgen receptor, follicle-stimulating hormone receptor, and anti-mullerian hormone type II receptor in single, isolated, human preantral follicles: Relevance to polycystic ovaries. *J Clin Endocrinol Metab.* 2007;92(3):1034–40.
109. Barbieri RL, Sluss PM, Powers RD, et al. Association of body mass index, age, and cigarette smoking with serum testosterone levels in cycling women undergoing in vitro fertilization. *Fertil Steril.* 2005;83(2):302–8.
110. Meldrum DR, Chang RJ, Giudice LC, et al. Role of decreased androgens in the ovarian response to stimulation in older women. *Fertil Steril.* 2013;99(1):5–11.
111. Richardson A, Jayaprakasan K. The Use of Androgen Priming in Women with Reduced Ovarian Reserve Undergoing Assisted Reproductive Technology. *Semin Reprod Med.* 2021;39(5–06):207–219.
112. Bosdou JK, Venetis CA, Kolibianakis EM, et al. The use of androgens or androgen-modulating agents in poor responders undergoing in vitro fertilization: A systematic review and meta-analysis. *Hum Reprod Update.* 2012;18(2):127–45.
113. Massin N, Cedrin-Durnerin I, Coussieu C, Galey-Fontaine J, Wolf JP, Hugues JN. Effects of transdermal testosterone application on the ovarian response to FSH in poor responders undergoing assisted reproduction technique—a prospective, randomized, double-blind study. *Hum Reprod.* 2006;21(5):1204–11.
114. Kim CH, Howles CM, Lee HA. The effect of transdermal testosterone gel pretreatment on controlled ovarian stimulation and IVF outcome in low responders. *Fertil Steril.* 2011;95(2):679–83.
115. Wiser A, Gonen O, Ghetler Y, Shavit T, Berkovitz A, Shulman A. Addition of dehydroepiandrosterone (DHEA) for poor-responder patients before and during IVF treatment improves the pregnancy rate: A randomized prospective study. *Hum Reprod.* 2010;25(10):2496–500.

116. Zhang Y, Zhang C, Shu J, et al. Adjuvant treatment strategies in ovarian stimulation for poor responders undergoing IVF: A systematic review and network meta-analysis. *Hum Reprod Update*. 2020;26(2):247–63.
117. Gleicher N, Kushnir VA, Albertini DF, Barad DH. Improvements in IVF in women of advanced age. *J Endocrinol*. 2016;230(1):F1–6.
118. Neves AR, Montoya-Botero P, Polyzos NP. The role of androgen supplementation in women with diminished ovarian reserve: Time to randomize, not meta-analyze. *Front Endocrinol*. 2021;12:653857.
119. Baerwald AR, Adams GP, Pierson RA. Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Hum Reprod Update*. 2012;18(1):73–91.
120. Baerwald A, Pierson R. Ovarian follicular waves during the menstrual cycle: Physiologic insights into novel approaches for ovarian stimulation. *Fertil Steril*. 2020;114(3):443–57.
121. Lu BJ, Lin CJ, Lin BZ, et al. ART outcomes following ovarian stimulation in the luteal phase: A systematic review and meta-analysis. *J Assist Reprod Genet*. 2021;38(8):1927–38.
122. Kuang Y, Chen Q, Hong Q, et al. Double stimulations during the follicular and luteal phases of poor responders in IVF/ICSI programmes (Shanghai protocol). *Reprod Biomed Online*. 2014;29(6):684–91.
123. Kalra K, Ratcliffe S, Gracia S, Martino CR, Coutifaris L, Barnhart C. Randomized controlled pilot trial of luteal phase recombinant FSH stimulation in poor responders. *Reprod Biomed Online*. 2008;17:745–50.
124. Rombauts L, Suikkari AM, MacLachlan V, Trounson AO, Healy DL. Recruitment of follicles by recombinant human follicle-stimulating hormone commencing in the luteal phase of the ovarian cycle. *Fertil Steril*. 1998;69:665–9.
125. Nazarenko TA, Martirosyan YO, Birukova AM, et al. Outcomes of ovarian stimulation in the follicular and luteal phases of the menstrual cycle in cancer patients. *Gynecol Endocrinol*. 2021;37(sup1):13–16.
126. Sunkara SK, Rittenberg V, Raine-Fenning N, et al. Association between the number of eggs and live birth in IVF treatment: An Analysis of 400 135 treatment cycles. *Hum Reprod*. 2011;26(7):1768–74.
127. Polat M, Mumusoglu S, Yarali Ozbek I, et al. Double or dual stimulation in poor ovarian responders: Where do we stand? *Ther Adv Reprod Health*. 2021;15:26334941211024172.
128. Hatirnaz S, Hatirnaz E, Ata B. Live birth following early follicular phase oocyte collection and vitrified-warmed embryo transfer 8 days later. *Reprod Biomed Online*. 2015;31(6):819–22.
129. Kwan I, Bhattacharya S, Kang A, Woolner A. Monitoring of stimulated cycles in assisted reproduction (IVF and ICSI). *Cochrane Database Syst Rev*. 2014;8:CD005289.

THE USE OF GONADOTROPIN-RELEASING HORMONE AGONISTS AND THE EFFICIENCY OF *IN VITRO* FERTILIZATION

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Introduction

Gonadotropin-releasing hormone (GnRH) is the primary hypothalamic regulator of reproductive function. The chemical structure of this compound was discovered in 1971 by a group of scientists in Andrew Schally's laboratory in New Orleans after they derived a small amount of GnRH from porcine hypothalamus [1, 2]. Roger Guillemin then characterized and independently synthesized the hormone, and he and Schally received the Nobel Prize for their achievements. GnRH is a decapeptide that is synthesized as part of a much larger precursor peptide, the GnRH-associated peptide. This peptide is composed of a sequence of 56 amino acids. The availability of the synthetic hormone for dynamic endocrine testing and receptor studies created new insights into the physiological role of GnRH in the hypothalamic–pituitary–gonadal axis [3].

GnRH is produced and released by a group of loosely connected neurons located in the medial basal hypothalamus, primarily within the arcuate nucleus, and in the preoptic area of the ventral hypothalamus. It is synthesized in the cell body, transported along the axons to the synapse, and released in a pulsatile fashion into the complex capillary net of the portal system of the pituitary gland [4]. GnRH binds selectively to the highly specific receptors of the anterior pituitary gonadotrophic cells and activates intracellular signalling pathways via the coupled G proteins, leading to the generation of several second messengers, including diacylglycerol and inositol-4,5-triphosphate. The former leads to activation of protein kinase C and the latter to the production of cyclic AMP and the release of calcium ions from intracellular pools [5–7]. Both events result in secretion and synthesis of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). A pulsatile GnRH release from the hypothalamus to the pituitary is required to ensure gonadotropin secretion [8–10]. In humans, the pulsatile release frequencies range from the shortest interpulse frequency of about 71 minutes in the late follicular phase to an interval of 216 minutes in the late luteal phase [11–13]. High frequency (>3 pulses/hour) and continuous exposure of GnRH to the pituitary failed to produce normal LH and FSH release [14–16] due to pituitary receptor desensitization. This mechanism is still not clear; however, we know that post-receptor signalling is involved and true receptor loss (downregulation) plays only an initial role in the process [17]. The pulsatile release by the GnRH neurons is likely based on an ultrashort feedback loop with GnRH itself; this autocrine process could serve as a timing mechanism to control the frequency of neurosecretion. Several mechanisms, based on calcium and cyclic AMP signalling, have been proposed to account for the pulse secretion. Another role of intracellular signalling in pulsatile generation has been suggested by the marked inhibition of Gi protein activation by LH, human chorionic gonadotropin (hCG), muscarine, oestradiol (E2), and GnRH levels [7, 18, 19].

After the discovery of the chemical structure of native GnRH type I, which proved to be the classic reproductive neuroendocrine factor, many were synthetically produced. Most were able to elicit a massive FSH and LH release from the pituitary and were therefore called GnRH agonists. However, under continuous administration of a GnRH agonist, both the synthesis and the subsequent release of LH, and to a lesser extent of FSH, became blocked (Figure 42.1). Other analogues by competitive receptor binding caused an immediate fall in pituitary gonadotropin secretion and were designated GnRH antagonists. In contrast to the agonistic compounds, the introduction of the GnRH antagonists into clinical practice has been hampered for a long time by problems concerning solubility and direct allergy-like side effects due to histamine release [20, 21]. These problems have now been resolved, leading to the third-generation GnRH antagonists. Two such drugs (ganirelix and cetrorelix) are routinely used during controlled ovarian stimulation (COS) protocols, and others such as elagolix (an oral GnRH antagonist, approved for the management of moderate to severe pain due to endometriosis) are under active investigation for use during IVF [22, 23]. The GnRH agonists have gained a wide range of clinical applications [24]. The main goal of using GnRH agonists is the achievement of suppression of the pituitary–ovarian (or testicular) axis for a limited or even an extended period of time.

Structural modifications

The elucidation of the structure, function, and metabolic pathways of native GnRH has prompted an intensive effort by research laboratories and the pharmaceutical industry to synthesize potent and longer-acting agonists and antagonists [25]. Over the past three decades, thousands of analogues of GnRH have been synthesized. Only seven of the agonistic analogues of GnRH have been approved and are in clinical use. The first major step in increasing the potency of GnRH was made with substitutions of glycine number 10 at the C terminus. Although 90% of the biologic activity is lost by the splicing of glycine number 10, most of it is restored with the attachment of NH₂-ethylamide to the proline at position 9, leading to nonapeptides [26]. The second major modification was the replacement of the glycine at position 6 by D-amino acids, which slows down enzymatic degradation. The combination of these two modifications was found to have synergistic biologic activity and proved to exhibit a higher receptor binding affinity. The affinity can be increased further by the introduction of larger, hydrophobic, and more lipophilic D-amino acids at position number 6. The increased lipophilic content is also associated with a prolonged half-life, which may be attributed to reduced renal excretion through increased plasma protein binding, or fat tissue storage of non-ionized, fat-soluble compounds [26]. For details about the structure, see Table 42.1.

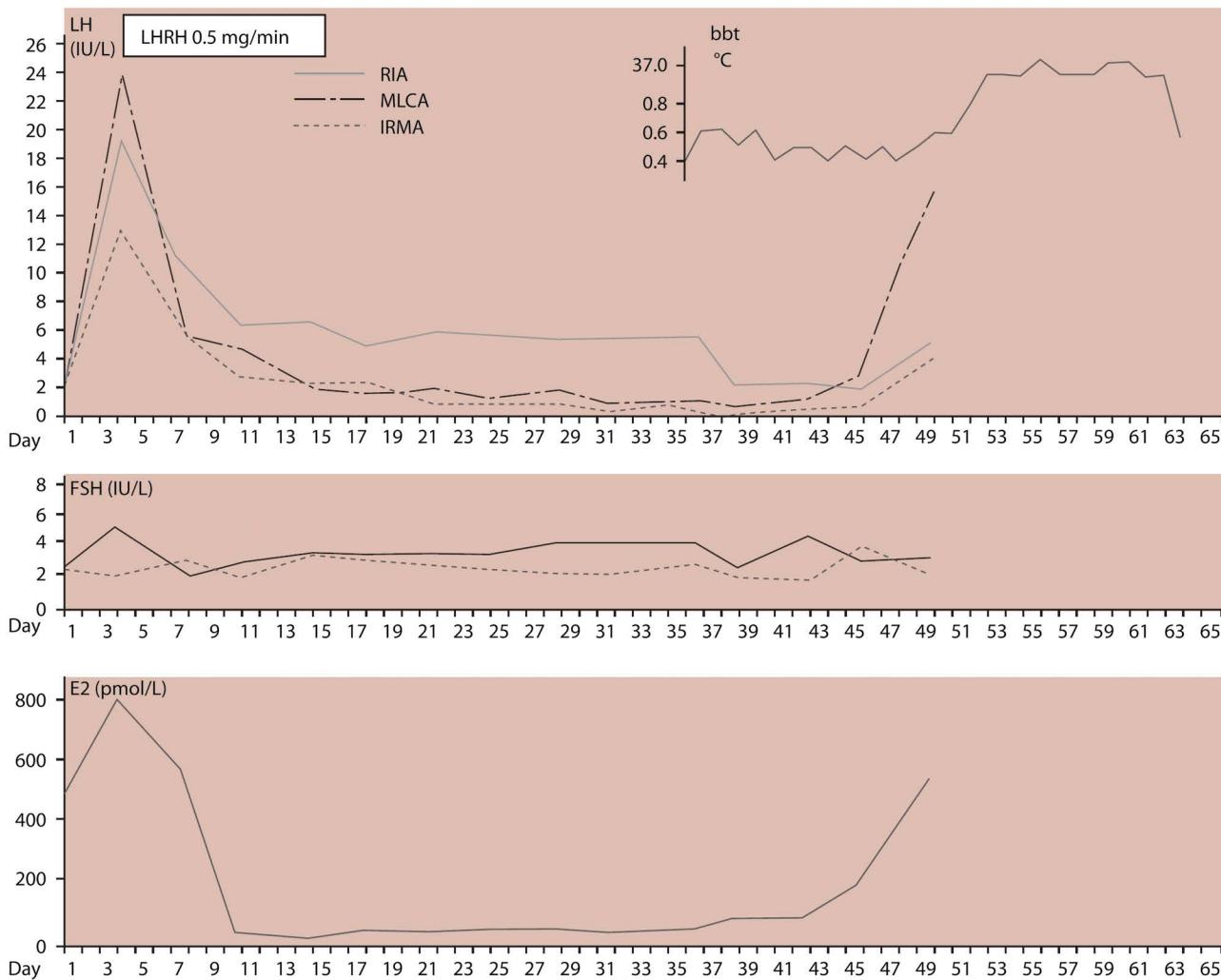


FIGURE 42.1 Hormone levels for LH, FSH, and E2 in a patient with continuous intravenous infusion of 0.5 mg/minute LHRH. LH was measured with three different assays and FSH with two different assays. Abbreviations: RIA, radioimmunoassay; MLCA, Magic Lite chemiluminescence assay; IRMA, immunoradiometric assay; bbt, basal body temperature; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, oestradiol; LHRH, luteinizing hormone-releasing hormone. (Courtesy of Prof. J. Schoemaker.)

TABLE 42.1 Amino Acid Sequence and Substitution of the Gonadotropin-Releasing Hormone (GnRH) Agonists

Compound						Position 6			Position 10		
Amino acid no	1	2	3	4	5	6	7	8	9	10	
Native GnRH	Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	GlyNH ₂	
Nonapeptides											
Leuprolide (Lupron, Lucrin), buserelin (Suprefact), goserelin (Zoladex), histrelin (Suprelin), deslorelin (Ovuplant)						Leu				N-Et-NH ₂	
							Ser(O'Bu)			N-Et-NH ₂	
							Ser(O'Bu)			AzaGlyNH ₂	
							D-His(Bzl)			AzaGlyNH ₂	
							D-Tr			N-Et-NH ₂	
Decapeptides											
Nafarelin (Synarel), triptorelin (Decapeptyl)						2Nal				GlyNH ₂	
							Trp			GlyNH ₂	

Clinical applications

The original goal for the development of agonistic analogues of GnRH was that they would eventually be used for the treatment of anovulation. However, soon after the elucidation of the structure of GnRH, the “paradoxical” ability of agonistic analogues to inhibit reproductive function in experimental animals was demonstrated [27]. The most important clinical applications of the potent GnRH agonists were derived from their capacity to cause rapid desensitization of the pituitary gland as a result of prolonged, non-pulsatile administration, leading to a decrease in serum gonadotropin levels and subsequently inhibition of ovarian steroidogenesis and follicular growth. The potential for reversibly inducing a state of hypogonadotropic hypogonadism, which was also termed “medical gonadectomy” or “medical hypophysectomy,” allowed for the relatively rapid and extensive introduction of GnRH agonists into clinical practice. For a variety of indications, complete abolition of gonadotropin secretion with subsequent suppression of gonadal steroids to the levels of castrated subjects was considered beneficial. This therapeutic approach already had its efficacy and merits proven in the treatment of metastatic prostatic cancer, breast cancer, central precocious puberty, endometriosis (including adenomyosis), uterine fibroids, hirsutism, and other conditions [28, 29].

Since the first report on the use of the combination of the GnRH agonist buserelin and gonadotropins for ovarian stimulation for *in vitro* fertilization (IVF) in 1984 [30], numerous studies have demonstrated the efficacy of this concept. Subsequently, the use of GnRH agonists has gained widespread popularity, and, for many years, the vast majority of assisted reproduction technology (ART) programmes used this approach as part of their primary protocol for COS prior to IVF. The major advantage initially offered by the agonists was the efficient abolition of the spontaneous LH surge [31]. The incidence of premature LH surges and subsequent luteinization in cycles with exogenous gonadotropin stimulation, without the use of a GnRH agonist, was observed by several investigators to range between 20% and 50%, leading to an increased cycle cancellation rate [32]. Moreover, a deleterious effect on both fertilization and pregnancy rates was noted [31, 33]. A meta-analysis of randomized controlled trials has shown that the use of GnRH agonists has not only reduced cancellation rates but also increased the number of oocytes and embryos, allowing better selection [34], so that, on average, the outcome in terms of pregnancy rates was improved [35].

More recently, several studies have compared the use of GnRH agonists with that of GnRH antagonists in ovarian stimulation protocols. Some studies have cited several advantages of using GnRH antagonists, including shorter duration of treatment, a reduction in the dose requirement of gonadotropin, and a lower incidence of ovarian hyperstimulation syndrome (OHSS) [36]. Interestingly, in the early days of using GnRH antagonist many argued that the GnRH agonist protocol produced higher clinical pregnancy rates and should therefore be favoured, particularly in good-prognosis patients who are not at high risk of severe OHSS [37]. However, as described later in this chapter, the use of GnRH antagonist has become much more common in ART, particularly for cases where the risk of OHSS is high, such as in a patient with polycystic ovarian syndrome (PCOS), thus allowing the use of GnRH agonist to “trigger” oocyte maturation with endogenous LH instead of hCG.

Remaining issues concerning the use of GnRH agonists in ART can be divided into the following four categories:

1. Which route of administration is the best?
2. Which agonist(s) should be used in ART?
3. What is the optimal dose?
4. What is the optimal scheme?

Which route of administration is the best?

Administration routes of GnRH agonists are intramuscular or subcutaneous depot injection, intranasal, or subcutaneous daily administration. Although there is an advantage for the patient in the single injection of the depot preparations, the duration of action is prolonged and rather unpredictable. The effect can last until the first weeks of pregnancy [38]. Broekmans et al. showed that rapid induction of a hypogonadotropic and hypogonadal state is possible in regularly cycling women by administration of a single depot of triptorelin. However, suppression of pituitary and ovarian function appears to be continued until the eighth week after the injection [38]. This is far longer than is actually needed. Devreker et al. found several negative effects of depot preparations, including a longer stimulation phase and consequently the need for more ampoules of gonadotropins, but more importantly they saw lower implantation and delivery rates (32.8% vs 21.1% and 48.9% vs 29.1%, respectively). Their conclusion was that since a long-acting GnRH agonist might interfere with the luteal phase and embryo development, short-acting GnRH agonists should be preferred in ART [39, 40].

A meta-analysis comparing depot versus daily administration concluded that there is no clear difference in pregnancy rate. Furthermore, the use of depot GnRH agonists is associated with increased gonadotropin requirements and longer stimulation periods and should therefore not be used based on cost-effectiveness [39]. Moreover, on a theoretical basis, it would be desirable to avoid any possible direct effect on the embryo, although several authors claim a normal outcome of pregnancy following inadvertent administration of a GnRH agonist during early pregnancy [41–46]. Lahat et al. reported a high incidence of attention-deficit/hyperactivity disorder in long-term follow-up of children inadvertently exposed to GnRH agonists early in pregnancy [47].

Thus, although depot preparations seem attractive because of their ease of administration for the patient, they should not be routinely used in IVF. One exception to this statement might be the prolonged use of GnRH analogues before IVF in patients with severe endometriosis, which is associated with higher ongoing pregnancy rates [48].

With the intranasal route, the absorption of the GnRH agonist fluctuates inter- and intra-individually, giving an unpredictable desensitization level, but usually this is sufficient to prevent premature LH surges. For research or study purposes, daily subcutaneous injections are preferred because of their more stable effect. The clinician has to strike a balance between comfort for the patient and a more stable effect in selecting the intranasal versus the subcutaneous route of administration.

Which agonist(s) should be used in ART?

Table 42.1 lists seven different GnRH agonists, but only four are commonly used in IVF programmes. An extensive search revealed only one article on the use of histrelin in IVF [49], while deslorelin has never been applied in human IVF. Except for its combination for the treatment of endometriosis, goserelin is not routinely used in ART, partly because it is only available as a

depot preparation. Other depot preparations on the market are triptorelin and leuprorelin acetate. Thirteen prospective randomized trials have compared different agonists with each other [50–61]. The problem with those studies is that the optimal dosage has not been determined for any of the applied individual agonists, and therefore the ability of these articles to answer the question of which compound should be used is limited. All the agonists seem effective and the differences in the studies can be explained by dosage incompatibility. These studies make absolutely clear that proper dose-finding studies for the use of GnRH agonists in ART are still urgently needed. It is obvious that the dose required for the prevention of premature LH surges during COS in ART will be different from that required to treat carcinoma of the prostate, which requires complete chemical castration (more on that follows).

What is the optimal dose?

Finding the right dose in the treatment of infertility disorders has been notoriously difficult. Because proper dose-finding studies for the use of gonadotropins were lacking, it took until the middle of the 1980s before an adequate treatment protocol, with a maximum of effect and a minimum of side effects, was introduced [62]. There is only one prospective, randomized, double-blind, placebo-controlled dose-finding study performed in IVF for the GnRH agonist triptorelin. This study demonstrated that the dosage needed for the suppression of the LH surge is much smaller than the dosage needed for the treatment of a malignant disease, namely, only 15%–50% [32]. It is very likely that dose-finding studies for the other agonists will give similar results. As per the recent literature, such studies have not been performed.

What is the optimal scheme?

Many treatment regimens with the use of GnRH agonists in ART have been designed. Initiation of the agonist treatment may be in either the early follicular or the mid-luteal phase of the preceding cycle. The cycle may be spontaneous or induced by progestogen and/or oestrogen compounds. There is still much debate about the optimal GnRH agonist protocol. Tan published a review article in 1994 stating that the so-called long protocol was superior to the short and ultrashort protocols [63]. Moreover, a major advantage of the long GnRH agonist protocol is its contribution to the planning of the ovum pick-up since both the initiation of exogenous gonadotropins after pituitary desensitization and the administration of hCG can be delayed without any detrimental effect on IVF outcome [64, 65]. A meta-analysis comparing ultrashort, short, and long IVF protocols showed a higher number of oocytes retrieved and higher pregnancy rates in the long protocol, although more ampoules of gonadotropins were needed [66]. In terms of gonadotropin suppression and numbers of retrieved oocytes, the mid-luteal phase of the preceding cycle is the optimal moment for the initiation of the GnRH agonist, in comparison to the follicular, early, or late luteal phases [67–69].

However, a problem with prospective randomized clinical studies is that certain groups of patients, such as poor responders (with or without elevated basal FSH) or patients with PCOS, are often excluded. There is a possibility that, especially in the excluded groups, other schemes are preferable. An unwanted side effect of starting the GnRH agonist in the luteal or follicular phase in the long protocol is the induction of the formation of functional cysts. Keltz et al. observed both a poor stimulation outcome and a reduction in pregnancy rates in a cycle with cyst formation [70]. However, Feldberg et al. could not confirm this

finding [71]. Ovarian cyst formation was reduced when pre-treatment with an oral contraceptive was applied [72]. Damario et al. showed the beneficial effect of this strategy in high responder patients with respect to cancellation rates and pregnancy rates [73]. A long GnRH agonist protocol in combination with an oral contraceptive seems to be advantageous in the prevention of functional ovarian cysts and especially for the larger IVF centres for programming of IVF cycles. Another practical advantage of including an oral contraceptive is preventing the coincidence of luteal GnRH agonist use with the possibility of an early pregnancy.

The mean desensitization phase with an agonist in the long protocols is about three weeks. Several investigators have tried to shorten this long duration of administration, leading to the so-called “early cessation protocol” [74–77]. Increased human menopausal gonadotropin/FSH requirements and cancellation rates were reported after early cessation in 137 normal IVF patients [77], but the opposite was found in a study that included 230 normally ovulating IVF patients [74], although pregnancy rates were the same in both studies [77]. The paradoxical drop of serum LH following early cessation that leads to significantly lower E2 levels on the day of hCG administration may have a deleterious effect on IVF outcome [74, 77]. The early discontinuation protocol may improve ovarian response based on a hypothetical effect on the ovary and was therefore additionally tested in poor responders. Although the number of retrieved oocytes was significantly higher and the amount of required gonadotropins was reduced after early cessation in comparison to the long protocol, this new approach reported no further advantages in these patients in terms of pregnancy and implantation rates [75, 76]. In conclusion, the currently available data do not favour an “early cessation” protocol, but this approach might have some beneficial effects in poor responders.

To prevent any detrimental effect of the profound suppression of circulating serum gonadotropins after cessation of GnRH agonist therapy, the opposite regimens have been developed in which the GnRH agonist administration is continued during the luteal phase, the so-called “continuous-long protocol.” In a large prospective randomized study ($n = 319$) comparing this continuous long protocol versus the standard long protocol, higher implantation and pregnancy rates were found in the continuous long protocol [78].

Since the use of a long protocol in poor responders has been found to result in reduced ovarian responses to hormonal stimulation, the short GnRH agonist protocol has been proposed as providing better stimulation for these patients. In the short or flare-up protocol, GnRH agonist therapy is started at cycle day 2 and gonadotropin treatment is started one day later. The immediate stimulatory action of the GnRH agonist serves as the initial stimulus for follicular recruitment (so-called “flare-up”). Adequate follicular maturation is on average reached in 10–12 days, which should allow enough time for sufficient pituitary desensitization to prevent any premature LH surges. The initial stimulatory effect of GnRH agonist on pituitary hormone levels may improve the ovarian response [79]. On the other hand, this short protocol might increase gonadotropins in the early phase, which induces enhanced ovarian androgen release. This is associated with lower oocyte quality and reduced ongoing pregnancy rates compared to the long protocol [80]. Nevertheless, experience to date shows that the short protocol has an important role in the treatment of poor responders [81]. Other investigators even promoted an “ultrashort protocol” in “poor responders,” in which the agonist is given over a period of three days in the early

follicular phase. On the second day of agonist administration, stimulation with gonadotropin administration (high dosages) is started [82]. Modifications to both the short [83, 84] and the long [85] protocols have been made in order to improve the response to COS in poor responders.

In very high responders or in patients at risk of OHSS, gonadotropin was discontinued whilst continuing the GnRH agonist; this so-called “coasting” was used in the past as prevention for the development of severe OHSS [86, 87]. This strategy allows a delay of a variable number of days in administering the hCG injection until safe E2 levels are attained. However, sufficient randomized controlled trials comparing coasting with no coasting are lacking [88]. Only one prospective comparative trial in 60 IVF patients showed a similar incidence of moderate and severe OHSS whether coasting was applied or not [89].

The most important advantages and disadvantages of the different GnRH agonist protocols are summarized in Table 42.2.

After the clinical availability of GnRH antagonists, an additional indication for the use of GnRH agonists became of interest. Indeed, today, GnRH analogues are effectively used as an alternative to hCG to “trigger” final oocyte maturation by causing the endogenous release of LH and FSH for the final maturation of the oocytes and ovulation [90, 91]. Since hCG is believed to contribute to the occurrence of OHSS, owing to its prolonged circulating half-life compared with native LH, this strategy is an attractive alternative for preventing OHSS. In the early 1990s, it was already shown that single-dose GnRH agonists administrated in COS-IVF patients were able to induce an endogenous rise in both LH and FSH levels, leading to follicular maturation and pregnancy [92, 93]. Mean serum LH and FSH levels rose over 4–12 hours and were elevated for 24–34 hours after GnRH agonist, in comparison to approximately six days of elevated hCG levels after 5000 IU hCG administration. The capacity for a single administration

of GnRH agonist to trigger follicular rupture in anovulatory women or in preparation for intrauterine insemination (IUI) has been well established. This seems to induce lower OHSS rates with comparable or even improved results, despite short luteal phases, in comparison to hCG cycles [90, 91, 94]. Interest in this approach was lost during the 1990s, because GnRH agonists were introduced in COS protocols to prevent premature luteinization by pituitary desensitization, precluding stimulation of the endogenous LH surge. However, interest has returned following the introduction of GnRH antagonist protocols in which the pituitary responsiveness is preserved [95]. This new concept of triggering final oocyte maturation after GnRH antagonist treatment by a single GnRH agonist injection was successfully tested in COS patients for IUI and in high responders for IVF [96]. None of these patients developed OHSS. The efficacy and success of this new treatment regimen was established in a prospective multicentre trial in which 47 patients were randomized to receive either 0.2 mg triptorelin, 0.5 mg leuprorelin, or 10,000 IU hCG [97]. The LH surges peaked at four hours after agonist administration and returned to baseline after 24 hours; the luteal-phase steroid levels were also closer to the physiologic range compared to the hCG groups. In terms of triggering the final stages of oocyte maturation, similar outcomes were observed in all groups, as demonstrated by the similar fertilization rates and oocyte quality [97].

A prospective randomized study in 105 stimulated IUI cycles treated with a GnRH antagonist in patients with clomiphene resistant PCOS showed statistically significant more clinical pregnancies after ovulation triggering by a GnRH agonist in comparison to hCG (28.2% vs 17.0% per completed cycle, respectively) [98]. Therefore, this new approach of ovulation triggering seems to be an attractive alternative to hCG in ART if administered in GnRH antagonist-treated cycles, with lower OHSS rates and similar or improved IVF outcomes.

TABLE 42.2 Summary of Advantages and Disadvantages of the Different Gonadotropin-Releasing Hormone (GnRH) Agonist Protocols

GnRH Agonist Protocol	Route of Administration	Administration Days of Cycle (CD)	Duration of Administration	Advantages	Disadvantages
Ultrashort protocol	IN/SC	CD 2, 3–4, 5	3 days	Patient's comfort	Low PR
Short protocol	IN/SC	CD 2, 3 until day of hCG	8–12 days	Patient's comfort	No programming
Long follicular	IN/SC	CD 2 until day of hCG	28–35 days	Programming, good PR	Long duration of administration
Long luteal	IN/SC	CD 21 until day of hCG	21–28 days	Programming, good PR	Long duration of administration
Menstrual early cessation	IN/SC	CD 21 until menses	7–12 days	Inconclusive	Low oestradiol levels
Follicular early cessation	IN/SC	CD 21 until stimulation day 6, 7	13–20 days	Inconclusive	Low oestradiol levels
Long follicular (depot)	Depot	CD 2	Once	Patient's comfort	(Too) long duration of action
Long luteal (depot)	Depot	CD 21	Once	Patient's comfort	(Too) long duration of action
Ultralong	IN/SC/depot	CD 2 or 21	8–12 weeks, depot two or three times	Only for special cases	Side effects due to oestrogen deficiency

Abbreviations: CD, cycle day; hCG, human chorionic gonadotropin; IN, intranasal; PR, pregnancy rate; SC, subcutaneous.

Conclusions

GnRH agonists are widely used in IVF to control the endogenous LH surge and to achieve augmentation of multi-follicular development. Disadvantages, such as the necessity for luteal support, increased total gonadotropin dose per treatment cycle, and consequently higher costs, appear to be outweighed by the observed increase in ability to control the cycle, the higher yield of good-quality oocytes and subsequently embryos, and the consequent improvement of pregnancy rates. The introduction of GnRH agonists in IVF is not an example of excellent research, since proper dose-finding studies are still awaited. Further research into finding the right dose and protocol could still improve the clinical benefits of the GnRH agonists. Initiatives to perform such studies are lacking. Daily administered short-acting preparations deserve preference to the depot formulations. Intranasal administration best fits a patient's comfort considerations, while the subcutaneous route may be advocated for research purposes. The long GnRH agonist protocols give the highest pregnancy rates in the normal responders. There is some evidence that the short flare-up protocol is the treatment of choice for patients with diminished ovarian reserve (poor responders). Dose reduction might be the key point in optimizing pregnancy rates. Finally, GnRH agonists can be used to induce final maturation and ovulation as an alternative to hCG in ART.

A recent review [99] compared the effects of conventional GnRH antagonist protocols with GnRH agonist protocols on IVF/ICSI outcomes in women with polycystic ovary syndrome (PCOS). The primary outcomes were live birth rate, ongoing pregnancy rate, and OHSS rate. This review showed that GnRH antagonist protocols as opposed to GnRH agonists led to a significantly lower OHSS rate, shorter stimulation duration (one day less), lower gonadotropin consumption, lower E2 levels on hCG day, but a lower number of retrieved oocytes. However, there were no significant differences in live birth rate, ongoing pregnancy rate, clinical pregnancy rate, and miscarriage rates. The authors concluded that conventional GnRH antagonist protocols represent a safer and cost-effective treatment choice for women with PCOS who are undergoing ART.

The efficiency of IVF

The use of ART procedures to treat infertile couples has significantly increased worldwide since its inception in the late 1970s. However, despite significant advancements in both clinical protocols for COS and in the embryology laboratory, the process of human reproduction has remained inefficient [100, 101]. By using the metric of number of live-born infants according to the number of embryos chosen for transfer, it has been demonstrated that over the years the majority of embryos produced during IVF cycles (about 85%) are wasted, since they fail to result in a live-born infant [102]. Furthermore, when the metric of live-born infants is calculated according to the number of oocytes retrieved (oocyte to baby rate), it has been demonstrated that over the years only about 5%–6% of the total oocytes collected and used result in a live-born infant. One of the critical challenges in the field remains the ability to identify competent embryos that are capable of becoming live-born infants. Women continue to be aggressively stimulated with high doses of gonadotropins with the goal of retrieving multiple oocytes to increase the number of embryos available for transfer. This approach, however, is associated with a number of risks, including OHSS, and increased cost due to the high doses of medications used. The use of GnRH agonists as a

replacement for the hCG ovulation trigger has helped to significantly decrease the risk of OHSS.

By examining IVF efficiency, according to age groups, between 2004 and 2013, the embryo wastage rate decreased across all ages, but particularly in younger women (under 35 years of age), for whom this rate decreased from 76.1% in 2004 to 65.2% in 2013 ($p < 0.001$) [102]. In the group of women over the age of 42 years, the embryo wastage rate only marginally decreased, and remained relatively high from 2004 to 2013 (98.0% to 97.2%, respectively). In this age group, there was also the smallest, albeit still significant ($p < 0.001$), change in the mean number of embryos transferred (3.3 in 2004 to 2.8 in 2013). Further data analysis showed that the average number of embryos transferred per year, averaged across all age groups, positively correlated with the embryo wastage rate (Spearman coefficient = 0.988, $p < 0.001$). In other words, as the number of embryos transferred decreased, the percentage of embryos wasted also decreased, without impacting the pregnancy rates. This pattern has been consistent since 1995 and is further proof that only a few embryos, if any, are competent for live birth per cohort in each ART cycle [103]. In conclusion, the decrease in observed embryo wastage rate is not due to an improved oocyte or embryo biology, but merely to a reduction in the mean number of embryos transferred (i.e. a smaller denominator in the equation of total live births divided by total number of embryos transferred).

References

1. Arimura A. The backstage story of the discovery of LHRH. *Endocrinology*. 1991;129:1687–9.
2. Schally AV, Nair RM, Redding TW, et al. Isolation of the luteinizing hormone and follicle-stimulating hormone-releasing hormone from porcine hypothalamus. *J Biol Chem*. 1971;246:7230–6.
3. Clayton RN, Catt KJ. Gonadotropin-releasing hormone receptors: Characterization, physiological regulation, and relationship to reproductive function. *Endocrinol Rev*. 1981;2:186–209.
4. Carmel PW, Araki S, Ferin M. Pituitary stalk portal blood collection in rhesus monkeys: Evidence for pulsatile release of gonadotropin-releasing hormone (GnRH). *Endocrinology*. 1976;99:243–8.
5. Stojilkovic SS, Reinhart J, Catt KJ. Gonadotropin-releasing hormone receptors: Structure and signal transduction pathways. *Endocrinol Rev*. 1994;15:462–99.
6. Kaiser UB, Conn PM, Chin WW. Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. *Endocrinol Rev*. 1997;18:46–70.
7. Catt KJ, Martinez-Fuentes AJ, Hu L, et al. Recent insights into the regulation of pulsatile GnRH secretion (abstract O-004). *Gynecol Endocrinol*. 2003;17:2.
8. Neill JD, Patton JM, Dailey RA, et al. Luteinizing hormone releasing hormone (LHRH) in pituitary stalk blood of rhesus monkeys: Relationship to level of LH release. *Endocrinology*. 1977;101:430–4.
9. Levine JE, Pau KY, Ramirez VD, et al. Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone release in unanesthetized, ovariectomized sheep. *Endocrinology*. 1982;111:1449–55.
10. Levine JE, Norman RL, Gliessman PM, et al. *In vivo* gonadotropin-releasing hormone release and serum luteinizing hormone measurements in ovariectomized, estrogen-treated rhesus macaques. *Endocrinology*. 1985;117:711–21.
11. Backstrom CT, McNeilly AS, Leask RM, et al. Pulsatile secretion of LH, FSH, prolactin, oestradiol and progesterone during the human menstrual cycle. *Clin Endocrinol*. 1982;17:29–42.
12. Reame N, Sauder SE, Kelch RP, et al. Pulsatile gonadotropin secretion during the human menstrual cycle: Evidence for altered frequency of gonadotropin-releasing hormone secretion. *J Clin Endocrinol Metab*. 1984;59:328–37.

13. Crowley WF Jr, Filicori M, Spratt DI, et al. The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. *Recent Prog Horm Res.* 1985;41:473–531.
14. Belchetz PE, Plant TM, Nakai Y, et al. Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone. *Science.* 1978;202:631–3.
15. Gharib SD, Wierman ME, Shupnik MA, et al. Molecular biology of the pituitary gonadotropins. *Endocrinol Rev.* 1990;11:177–99.
16. Nillius SJ, Wide L. Variation in LH and FSH response to LH-releasing hormone during the menstrual cycle. *J Obstet Gynaecol Br Common.* 1972;79:865–73.
17. Conn PM, Crowley WF Jr. Gonadotropin-releasing hormone and its analogs. *Annu Rev Med.* 1994;45:391–405.
18. Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, et al. Local regulation of gonadotroph function by pituitary gonadotropin-releasing hormone. *Endocrinology.* 2000;141:1187–95.
19. Merchenthaler I. Identification of estrogen receptor-B in the GnRH neurons of the rodent hypothalamus (abstract O-024). *Gynecol Endocrinol.* 2003;17:12.
20. Reissmann T, Diedrich K, Comaru-Schally AM, et al. Introduction of LHRH-antagonists into the treatment of gynaecological disorders. *Hum Reprod.* 1994;9:769.
21. Gordon K, Hodgen GD. Will GnRH antagonists be worth the wait? *Reprod Med Rev.* 1992;1:189–94.
22. Huirne JA, Lambalk CB. Gonadotropin-releasing hormone-receptor antagonists. *Lancet.* 2001;358:1793–803.
23. Ng J, Chwalisz K, Carter DC, Klein CE. Dose-dependent suppression of gonadotropins and ovarian hormones by elagolix in healthy premenopausal women. *J Clin Endocrinol Metab.* 2017;102:1683–91.
24. Andreyko JL, Marshall LA, Dumesic DA, et al. Therapeutic uses of gonadotropin-releasing hormone analogs. *Obstet Gynecol Surv.* 1987;42:1–21.
25. Nestor JJ Jr. Developments of agonistic LHRH analogs. In: *LHRH and Its Analogs.* Vickery BH, Nestor JJ, Jr., Hafez ESE (eds.). Lancaster, UK: MTP Press, pp. 3–15, 1984.
26. Karten MJ, Rivier JE. Gonadotropin-releasing hormone analog design. Structure–function studies toward the development of agonists and antagonists: Rationale and perspective. *Endocrinol Rev.* 1986;7:44–66.
27. Corbin A, Beattie CW. Post-coital contraceptive and uterotrophic effects of luteinizing hormone releasing hormone. *Endocrinol Res Commun.* 1975;2:445–58.
28. Conn PM, Crowley WF Jr. Gonadotropin-releasing hormone and its analogs. *N Engl J Med.* 1991;324:93–103.
29. Klijn JGM. LHRH-agonist therapy in breast cancer (abstract O-016). *Gynecol Endocrinol.* 2003;17:8.
30. Porter RN, Smith W, Craft IL, et al. Induction of ovulation for *in vitro* fertilisation using buserelin and gonadotropins. *Lancet.* 1984;2:1284–5.
31. Fleming R, Coutts JR. Induction of multiple follicular growth in normally menstruating women with endogenous gonadotropin suppression. *Fertil Steril.* 1986;45:226–30.
32. Janssens RM, Lambalk CB, Vermeiden JP, et al. Dose finding study of triptorelin acetate for prevention of a premature LH surge in IVF: A prospective, randomized, double-blind, placebo-controlled study. *Hum Reprod.* 2000;15:2333–40.
33. Loumaye E. The control of endogenous secretion of LH by gonadotropin-releasing hormone agonists during ovarian hyperstimulation for *in vitro* fertilization and embryo transfer. *Hum Reprod.* 1990;5:357–76.
34. Templeton A, Morris JK. Reducing the risk of multiple births by transfer of two embryos after *in vitro* fertilization. *N Engl J Med.* 1998;339:573–7.
35. Hughes EG, Fedorkow DM, Daya S, et al. The routine use of gonadotropin-releasing hormone agonists prior to *in vitro* fertilization and gamete intrafallopian transfer: A meta-analysis of randomized controlled trials. *Fertil Steril.* 1992;58:888–96.
36. Al-Inany HG, Youssef MAFM, Aboulghar M, et al. Gonadotrophin releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev.* 2011;5:CD001750.
37. Orvieto R, Patrizio P. GnRH agonist versus GnRH antagonist in ovarian stimulation: An ongoing debate. *Reprod Biomed Online.* 2013;26:4–8.
38. Broekmans FJ, Bernardus RE, Berkhouit G, et al. Pituitary and ovarian suppression after early follicular and mid-luteal administration of a LHRH agonist in a depot formulation: Decapeptyl CR. *Gynecol Endocrinol.* 1992;6:153–61.
39. Devreker F, Govaerts I, Bertrand E, et al. The longacting gonadotropin-releasing hormone analogues impaired the implantation rate. *Fertil Steril.* 1996;65:122–6.
40. Albuquerque LE, Saconato H, Maciel MC. Depot versus daily administration of gonadotrophin releasing hormone agonist protocols for pituitary desensitization in assisted reproduction cycles. *Cochrane Database Syst Rev.* 2002;3:CD002808.
41. Weissman A, Shoham Z. Favorable pregnancy outcome after administration of a long-acting gonadotrophin-releasing hormone agonist in the mid-luteal phase. *Hum Reprod.* 1993;8:496–7.
42. Balasch J, Martinez F, Jove I, et al. Inadvertent gonadotrophin-releasing hormone agonist (GnRHa) administration in the luteal phase may improve fecundity in *in vitro* fertilization patients. *Hum Reprod.* 1993;8:1148–51.
43. Ron-El R, Lahat E, Golan A, et al. Development of children born after ovarian superovulation induced by long-acting gonadotropin-releasing hormone agonist and menotropins, and by *in vitro* fertilization. *J Pediatr.* 1994;125:734–7.
44. Cahill DJ, Fountain SA, Fox R, et al. Outcome of inadvertent administration of a gonadotrophin-releasing hormone agonist (buserelin) in early pregnancy. *Hum Reprod.* 1994;9:1243–6.
45. Gartner B, Moreno C, Marinaro A, et al. Accidental exposure to daily long-acting gonadotrophin-releasing hormone analogue administration and pregnancy in an *in vitro* fertilization cycle. *Hum Reprod.* 1997;12:2557–9.
46. Taskin O, Gokdeniz R, Atmaca R, et al. Normal pregnancy outcome after inadvertent exposure to long acting gonadotrophin-releasing hormone agonist in early pregnancy. *Hum Reprod.* 1999;14:1368–71.
47. Lahat E, Raziel A, Friedler S, et al. Long-term follow-up of children born after inadvertent administration of a gonadotrophin-releasing hormone agonist in early pregnancy. *Hum Reprod.* 1999;14:2656–60.
48. Surrey ES, Silverberg KM, Surrey MW, et al. Effect of prolonged gonadotropin-releasing hormone agonist therapy on the outcome of *in vitro* fertilization–embryo transfer in patients with endometriosis. *Fertil Steril.* 2002;78:699–704.
49. de Ziegler D, Cedars MI, Randle D, et al. Suppression of the ovary using a gonadotropin releasing-hormone agonist prior to stimulation for oocyte retrieval. *Fertil Steril.* 1987;48:807–10.
50. Balasch J, Jove IC, Moreno V, et al. The comparison of two gonadotropin-releasing hormone agonists in an *in vitro* fertilization program. *Fertil Steril.* 1992;58:991–4.
51. Parinaud J, Oustry P, Perineau M, et al. Randomized trial of three luteinizing hormone-releasing hormone analogues used for ovarian stimulation in an *in vitro* fertilization program. *Fertil Steril.* 1992;57:1265–8.
52. Penzias AS, Shamma FN, Gutmann JN, et al. Nafarelin versus leuprolide in ovulation induction for *in vitro* fertilization: A randomized clinical trial. *Obstet Gynecol.* 1992;79:739–42.
53. Tapanainen J, Hovatta O, Juntunen K, et al. Subcutaneous goserelein versus intranasal buserelin for pituitary down-regulation in patients undergoing IVF: A randomized comparative study. *Hum Reprod.* 1993;8:2052–5.
54. Dantas ZN, Vicino M, Balmaceda JP, et al. Comparison between nafarelin and leuprolide acetate for *in vitro* fertilization: Preliminary clinical study. *Fertil Steril.* 1994;61:705–8.

55. Goldman JA, Dicker D, Feldberg D, et al. A prospective randomized comparison of two gonadotrophin releasing hormone agonists, nafarelin acetate and buserelin acetate, in *in vitro* fertilization–embryo transfer. *Hum Reprod.* 1994;9:226–8.
56. Tarlatzis BC, Grimbizis G, Pournaropoulos F, et al. Evaluation of two gonadotropin-releasing hormone (GnRH) analogues (leuprolide and buserelin) in short and long protocols for assisted reproductive techniques. *J Assist Reprod Genet.* 1994;11: 85–91.
57. Lockwood GM, Pinkerton SM, Barlow DH. A prospective randomized single-blind comparative trial of nafarelin acetate with buserelin in long-protocol gonadotrophin-releasing hormone analogue controlled *in vitro* fertilization cycles. *Hum Reprod.* 1995;10:293–8.
58. Tanos V, Friedler S, Shushan A, et al. Comparison between nafarelin acetate and D-Trp⁶-LHRH for temporary pituitary suppression in *in vitro* fertilization (IVF) patients: A prospective crossover study. *J Assist Reprod Genet.* 1995;12:715–9.
59. Oyesanya OA, Teo SK, Quah E, et al. Pituitary downregulation prior to *in vitro* fertilization and embryo transfer: A comparison between a single dose of Zoladex depot and multiple daily doses of Suprefact. *Hum Reprod.* 1995;10:1042–4.
60. Avrech OM, Goldman GA, Pinkas H, et al. Intranasal nafarelin versus buserelin (short protocol) for controlled ovarian hyperstimulation before *in vitro* fertilization: A prospective clinical trial. *Gynecol Endocrinol.* 1996;10:165–70.
61. Corson SL, Gutmann JN, Batzer FR, et al. A doubleblind comparison of nafarelin and leuprolide acetate for down-regulation in IVF cycles. *Int J Fertil Menopausal Stud.* 1996;41:446–9.
62. Polson DW, Mason HD, Saldahna MB, et al. Ovulation of a single dominant follicle during treatment with low-dose pulsatile follicle stimulating hormone in women with polycystic ovary syndrome. *Clin Endocrinol.* 1987;26:205–12.
63. Tan SL. Luteinizing hormone-releasing hormone agonists for ovarian stimulation in assisted reproduction. *Curr Opin Obstet Gynecol.* 1994;6:166–72.
64. Chang SY, Lee CL, Wang ML, et al. No detrimental effects in delaying initiation of gonadotropin administration after pituitary desensitization with gonadotropin-releasing hormone agonist. *Fertil Steril.* 1993;59:183–6.
65. Dimitry ES, Oskarsson T, Conaghan J, et al. Beneficial effects of a 24 h delay in human chorionic gonadotrophin administration during *in vitro* fertilization treatment cycles. *Hum Reprod.* 1991;6:944–6.
66. Daya S. Gonadotrophin-releasing hormone agonist protocols for pituitary desensitization in *in vitro* fertilization and gamete intrafallopian transfer cycles. *Cochrane Database Syst Rev.* 2000;2:CD001299.
67. Pellicer A, Simon C, Miro F, et al. Ovarian response and outcome of *in vitro* fertilization in patients treated with gonadotrophin-releasing hormone analogues in different phases of the menstrual cycle. *Hum Reprod.* 1989;4:285–9.
68. Kondaveeti-Gordon U, Harrison RF, Barry-Kinsella C, et al. A randomized prospective study of early follicular or midluteal initiation of long protocol gonadotropin-releasing hormone in an *in vitro* fertilization program. *Fertil Steril.* 1996;66: 582–6.
69. San Roman GA, Surrey ES, Judd HL, et al. A prospective randomized comparison of luteal phase versus concurrent follicular phase initiation of gonadotropin-releasing hormone agonist for *in vitro* fertilization. *Fertil Steril.* 1992;58:744–9.
70. Keltz MD, Jones EE, Duleba AJ, et al. Baseline cyst formation after luteal phase gonadotropin-releasing hormone agonist administration is linked to poor *in vitro* fertilization outcome. *Fertil Steril.* 1995;64:568–72.
71. Feldberg D, Ashkenazi J, Dicker D, et al. Ovarian cyst formation: A complication of gonadotropin-releasing hormone agonist therapy. *Fertil Steril.* 1989;51:42–5.
72. Biljan MM, Mahutte NG, Dean N, et al. Pretreatment with an oral contraceptive is effective in reducing the incidence of functional ovarian cyst formation during pituitary suppression by gonadotropin-releasing hormone analogues. *J Assist Reprod Genet.* 1998;15:599–604.
73. Damario MA, Barmat L, Liu HC, et al. Dual suppression with oral contraceptives and gonadotrophin releasing hormone agonists improves *in vitro* fertilization outcome in high responder patients. *Hum Reprod.* 1997;12:2359–65.
74. Cedrin-Durnerin I, Bidart JM, Robert P, et al. Consequences on gonadotrophin secretion of an early discontinuation of gonadotropin-releasing hormone agonist administration in short-term protocol for *in vitro* fertilization. *Hum Reprod.* 2000;15: 1009–14.
75. Dirnfeld M, Fruchter O, Yshai D, et al. Cessation of gonadotropin-releasing hormone analogue (GnRH-a) upon down-regulation versus conventional long GnRH-a protocol in poor responders undergoing *in vitro* fertilization. *Fertil Steril.* 1999;72:406–11.
76. Garcia-Velasco JA, Isaza V, Requena A, et al. High doses of gonadotrophins combined with stop versus non-stop protocol of GnRH analogue administration in low responder IVF patients: A prospective, randomized, controlled trial. *Hum Reprod.* 2000;15: 2292–6.
77. Fujii S, Sagara M, Kudo H, et al. A prospective randomized comparison between long and discontinuous-long protocols of gonadotropin-releasing hormone agonist for *in vitro* fertilization. *Fertil Steril.* 1997;67:1166–8.
78. Fujii S, Sato S, Fukui A, et al. Continuous administration of gonadotropin-releasing hormone agonist during the luteal phase in IVF. *Hum Reprod.* 2001;16:1671–5.
79. Padilla SL, Dugan K, Maruschak V, et al. Use of the flare-up protocol with high dose human follicle stimulating hormone and human menopausal gonadotropins for *in vitro* fertilization in poor responders. *Fertil Steril.* 1996;65:796–9.
80. Loumaye E, Coen G, Pamper S, et al. Use of a gonadotropin-releasing hormone agonist during ovarian stimulation leads to significant concentrations of peptide in follicular fluids. *Fertil Steril.* 1989;52:256–63.
81. Fasouliotis SJ, Simon A, Laufer N. Evaluation and treatment of low responders in assisted reproductive technique: A challenge to meet. *J Assist Reprod Genet.* 2000;17:357–73.
82. Acharya U, Irvine S, Hamilton M, et al. Prospective study of short and ultrashort regimens of gonadotropin-releasing hormone agonist in an *in vitro* fertilization program. *Fertil Steril.* 1992;58:1169–73.
83. Scott RT, Navot D. Enhancement of ovarian responsiveness with microdoses of gonadotropin-releasing hormone agonist during ovulation induction for *in vitro* fertilization. *Fertil Steril.* 1994;61:880–5.
84. Surrey ES, Bower J, Hill DM, et al. Clinical and endocrine effects of a microdose GnRH agonist flare regimen administered to poor responders who are undergoing *in vitro* fertilization. *Fertil Steril.* 1998;69:419–24.
85. Feldberg D, Farhi J, Ashkenazi J, et al. Minidose gonadotropin-releasing hormone agonist is the treatment of choice in poor responders with high follicle-stimulating hormone levels. *Fertil Steril.* 1994;62:343–6.
86. Sher G, Zouves C, Feinman M, et al. “Prolonged coasting”: An effective method for preventing severe ovarian hyperstimulation syndrome in patients undergoing *in vitro* fertilization. *Hum Reprod.* 1995;10:3107–9.
87. Fluker MR, Hooper WM, Yuzpe AA. Withholding gonadotropins (“coasting”) to minimize the risk of ovarian hyperstimulation during superovulation and *in vitro* fertilization-embryo transfer cycles. *Fertil Steril.* 1999;71:294–301.
88. D’Angelo A, Amso N. “Coasting” (withholding gonadotrophins) for preventing ovarian hyperstimulation syndrome. *Cochrane Database Syst Rev.* 2002;6:CD002811.

89. Egbase PE, Sharhan MA, Grudzinskas JG. Early unilateral follicular aspiration compared with coasting for the prevention of severe ovarian hyperstimulation syndrome: A prospective randomized study. *Hum Reprod.* 1999;14:1421–5.
90. Emperaire JC, Ruffie A. Triggering ovulation with endogenous luteinizing hormone may prevent the ovarian hyperstimulation syndrome. *Hum Reprod.* 1991;6:506–10.
91. Lanzone A, Fulghesu AM, Villa P, et al. Gonadotropin-releasing hormone agonist versus human chorionic gonadotropin as a trigger of ovulation in polycystic ovarian disease gonadotropin hyperstimulated cycles. *Fertil Steril.* 1994;62:35–41.
92. Gonen Y, Balakier H, Powell W, et al. Use of gonadotropin-releasing hormone agonist to trigger follicular maturation for *in vitro* fertilization. *J Clin Endocrinol Metab.* 1990;71:918–22.
93. Itskovitz J, Boldes R, Levron J, et al. Induction of preovulatory luteinizing hormone surge and prevention of ovarian hyperstimulation syndrome by gonadotropin-releasing hormone agonist. *Fertil Steril.* 1991;56:213–20.
94. Romeu A, Monzo A, Peiro T, et al. Endogenous LH surge versus hCG as ovulation trigger after low-dose highly purified FSH in IUI: A comparison of 761 cycles. *J Assist Reprod Genet.* 1997;14: 518–24.
95. Olivennes F, Fanchin R, Bouchard P, et al. Triggering of ovulation by a gonadotropin-releasing hormone (GnRH) agonist in patients pretreated with a GnRH antagonist. *Fertil Steril.* 1996;66: 151–3.
96. Itskovitz-Eldor J, Kol S, Mannaerts B. Use of a single bolus of GnRH agonist triptorelin to trigger ovulation after GnRH antagonist ganirelix treatment in women undergoing ovarian stimulation for assisted reproduction, with special reference to the prevention of ovarian hyperstimulation syndrome: Preliminary report: Short communication. *Hum Reprod.* 2000;15:1965–8.
97. Fauser BC, de Jong D, Olivennes F, et al. Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for *in vitro* fertilization. *J Clin Endocrinol Metab.* 2002;87:709–15.
98. Egbase PE, Grudzinskas JG, Al Sharhan M, Ashkenazi L. hCG or GnRH agonist to trigger ovulation in GnRH antagonist-treated intrauterine insemination cycles: A prospective randomized study. *Hum Reprod.* 2002;17:2-O-006.
99. Kadoura S, Alhalabi M, Nattouf AH. Conventional GnRH antagonist protocols versus long GnRH agonist protocol in IVF/ICSI cycles of polycystic ovary syndrome women: A systematic review and meta-analysis. *Sci Rep.* 2022;12:4456.
100. Patrizio P, Bianchi V, Lalioti MD, Gerasimova T, Sakkas D. High rate of biological loss in assisted reproduction: It is in the seed, not in the soil. *Reprod Biomed Online.* 2007;14:92–5.
101. Bromer JG, Sakkas D, Siano LJ, Benadiva CA, Patrizio P. Reproductive efficiency of women over the age of 40 and the low risk of multiple pregnancies. *Reprod Biomed Online.* 2009;19(Suppl 4): 4316.
102. Ghazal S, Patrizio P. Embryo wastage rates remain high in assisted reproductive technology (ART): A look at the trends from 2004–2013 in the United States. *JARG.* 2017;34(2):159–66.
103. Kovalevsky G, Patrizio P. High rates of embryo wastage with use of assisted reproductive technology: A look at the trends between 1995 and 2001 in the United States. *Fertil Steril.* 2005;84:325–30.

GONADOTROPIN-RELEASING HORMONE ANTAGONISTS IN OVARIAN STIMULATION FOR *IN VITRO* FERTILIZATION

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Introduction

Although the first baby born after *in vitro* fertilization was conceived in a non-stimulated cycle [1], it was soon accepted that the role of IVF, as an efficient therapeutic modality for sub-fertile couples, could only be served through multi-follicular development, achieved with the use of gonadotropins [2]. Gonadotropin use, however, was frequently associated with premature luteinizing hormone surge prior to oocyte retrieval, which led to cycle cancellation in approximately one out of five women [3, 4].

The problem of the premature LH surge was managed by the introduction of gonadotropin-releasing hormone (GnRH) antagonists in ovarian stimulation [5], thanks to the pioneering work of Schally et al. and Guillemain et al. in the early 1970s [6, 7]. Both GnRH agonists and antagonists were available in the early 1980s for suppression of endogenous LH secretion. GnRH antagonists, however, could not be used for this purpose due to the associated allergic reactions provoked by their administration [8], leaving GnRH agonists as the only available choice.

Following pituitary downregulation by GnRH agonists and avoidance of a premature LH surge, unhindered use of gonadotropins in ovarian stimulation led to the collection of more oocytes and to an increase in the number of good-quality embryos available for transfer [9]. This was associated with an increase in pregnancy rates compared to cycles where no suppression of a premature LH surge was performed, as shown by one of the first meta-analyses in reproductive medicine [10].

The use of GnRH agonists became universal through the 1980s, 1990s, and until the early 2000s, characterizing IVF throughout this period as the GnRH agonist era [11]. However, there is probably not much doubt that if GnRH antagonist use had not been associated with allergic reactions, they would have been adopted as the analogue of choice instead of GnRH agonists. This is mainly due to the fact that GnRH antagonist action starts immediately after their administration as opposed to the lengthy downregulation period required with GnRH agonists. In addition, GnRH agonist use is associated with oestrogen deprivation symptoms during the downregulation period, the occurrence of ovarian cysts at initiation of stimulation, and ovarian hyperstimulation syndrome (OHSS) following human chorionic gonadotropin (hCG) administration.

For the preceding reasons, the introduction in the early 2000s of the third generation of GnRH antagonists, which lacked histamine release problems and thus did not lead to allergic reactions [12, 13], was perceived by the scientific community as a great opportunity to simplify and optimize ovarian stimulation.

GnRH agonists versus GnRH antagonists

The introduction of GnRH antagonists was followed by an initial period of debate regarding their comparative efficacy with GnRH

agonists. This was fuelled by several conflicting meta-analyses in favour [14] or against [15, 16] their use.

Currently, there is no difference in the probability of live birth between GnRH antagonists and GnRH agonists (OR 1.02, 95% CI 0.85–1.23) [17] in the general IVF population and in poor responder patients (RR 0.89 95% CI 0.56–1.41) [18], whereas a lower probability of OHSS is associated with the use of GnRH antagonists in the general IVF population (OR 0.61, 95% CI 0.51–0.72) [17]. Based on this data, the latest European Society of Human Reproduction and Embryology (ESHRE) guideline on ovarian stimulation strongly recommends the use of GnRH antagonist over GnRH agonist protocols [19].

GnRH antagonists make ovarian stimulation more patient friendly, requiring fewer days of treatment compared to GnRH agonists [14]. In addition, they constitute a more rational way to inhibit a premature LH rise compared to GnRH agonists, which need to be administered for this purpose approximately three weeks before the LH rise is likely to occur.

Based on data from the German Registry (2001–2017), the use of GnRH antagonists in everyday practice shows an impressive clear trend for replacing GnRH agonists as the analogue of choice for suppressing the premature LH rise (Deutsches IVF Register. DIR Jahrbuch. 2000–2017. <http://www.deutsches-ivf-register.de>) (Figure 43.1).

Type, scheme, dose, and timing of GnRH antagonist administration

Two types of third-generation GnRH antagonists have been developed: ganirelix (Organon, Oss, The Netherlands) [20] and cetrorelix (ASTA-Medica, Frankfurt, Germany) [21].

The majority of GnRH antagonist cycles performed today follow the daily-dose scheme [22], although in the past a single-dose antagonist scheme has been used [23].

On the basis of dose-finding studies, the optimal dose for the daily dose GnRH antagonist scheme is 0.25 mg for both cetrorelix and ganirelix [24, 25].

The incidence of premature LH surge in patients undergoing ovarian stimulation was similar between the two antagonistic analogues in phase III trials, however, no conclusive direct comparison between Ganirelix and Cetrorelix for ovarian stimulation for IVF has so far been performed [14].

GnRH antagonists can be initiated in either a fixed or a flexible protocol. In the fixed protocol, antagonist initiation occurs on a certain stimulation day on which it is assumed that the LH rise becomes imminent in the majority of patients. In the early introductory GnRH antagonist studies, the LH rise was thought to become imminent on day 6 of stimulation [26], while in the more recent studies [25], this is believed to occur on day 5 of stimulation [27].

In a flexible GnRH antagonist protocol, the antagonist is administered only after certain endocrine and/or sonographic

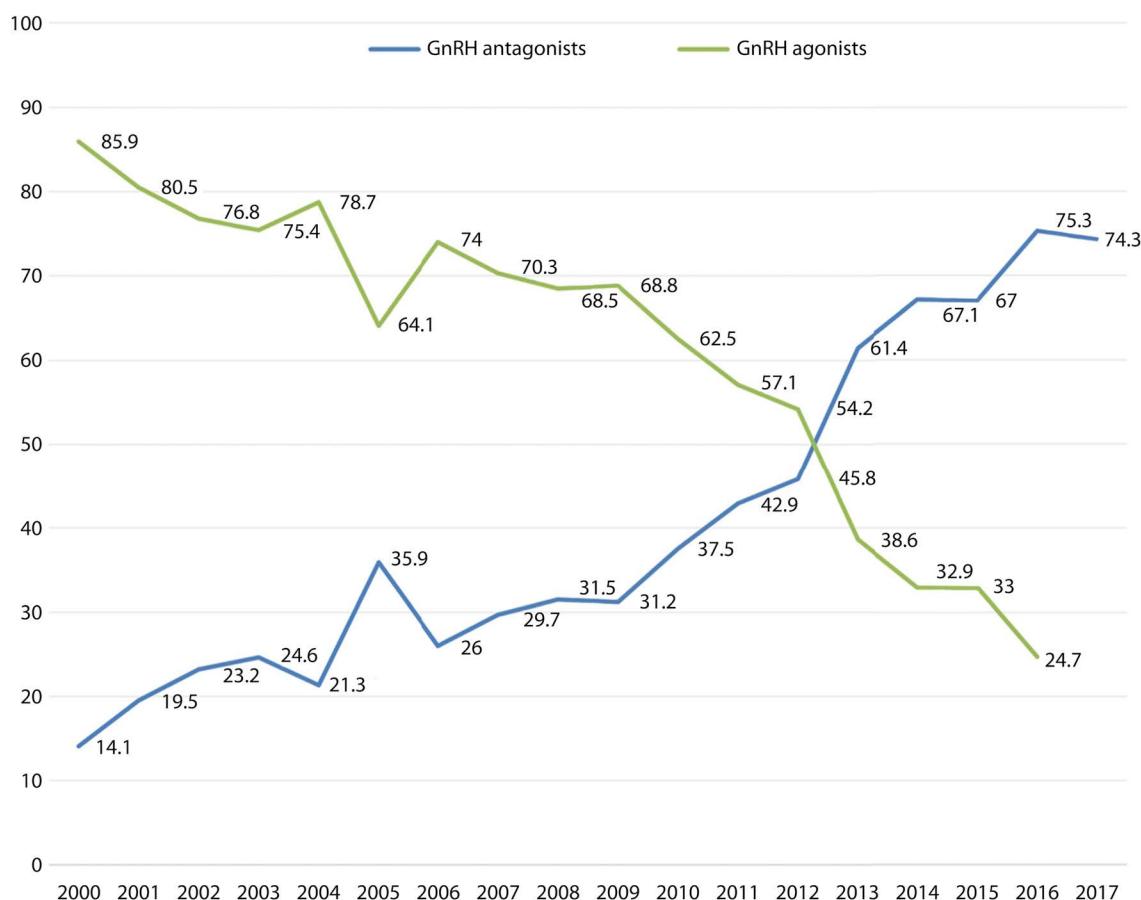


FIGURE 43.1 Proportion of cycles performed with either GnRH agonists or antagonists based on data published by the Deutsches IVF Register (DIR) (2000–2017). Abbreviation: GnRH, gonadotropin-releasing hormone.

criteria indicating a risk for a LH rise are present. These criteria have differed between studies [28]. A large variation in the criteria used in the flexible protocol is present in the literature [29] (Figure 43.2).

Apparently, fixed GnRH antagonist initiation is a simpler protocol that requires less monitoring compared to the flexible one. However, flexible GnRH antagonist administration might avoid unnecessary analogue use, when no follicle is present on day 5 of stimulation and thus LH rise is unlikely.

Four randomized controlled trials (RCTs) have been published comparing fixed versus flexible GnRH antagonist administration in patients undergoing IVF [28, 30–32]. A stratified analysis of these RCTs suggests that no significant difference appears to exist in clinical pregnancy rates (risk ratio = 0.85, 95% CI 0.65–1.11).

Following that meta-analysis, three additional RCTs comparing the fixed versus the flexible GnRH antagonist protocol have been performed in normovulatory patients [33], in patients with PCOS [34], and in patients with predicted high ovarian response [35], showing no significant difference in the probability of pregnancy.

However, it is important to note that in the RCTs on fixed versus flexible protocols, only a fraction of the patients randomized to the flexible approach indeed had a later initiation of GnRH antagonists, and, accordingly, the true effect of delayed GnRH antagonist initiation has not been precisely determined in these trials.

Programming the initiation of a GnRH antagonist cycle

In a GnRH antagonist cycle, initiation of gonadotropin stimulation is dependent on the occurrence of menstruation. In contrast, in a long luteal GnRH agonist protocol, initiation of stimulation is more flexible, since it occurs 10–15 days following menstruation, when downregulation is confirmed. However, if deemed necessary, it can be postponed for a number of days. In both GnRH agonist and antagonist cycles, knowing the type and length of patients' cycles makes it feasible to avoid the concomitant initiation of an excessive number of IVF cycles that can increase a centre's workload beyond what is considered manageable.

On the other hand, there have been efforts to program the initiation of an IVF cycle in order to prevent the occurrence of oocyte retrievals on Sundays or on weekends. This is a challenging task for both GnRH agonist and GnRH antagonist cycles, since duration of stimulation is characterized by a significant inter- and even intra-individual variation [26, 36, 37]. In GnRH antagonist cycles, sex steroid pre-treatment has been used for this purpose in the form of oral contraceptive pill (OCP) pre-treatment for 14–28 days before initiation of stimulation [38].

However, this strategy has been associated with a decreased probability of ongoing pregnancy. A systematic review and meta-analysis pooling results from 6 RCTs, including 1335 patients,

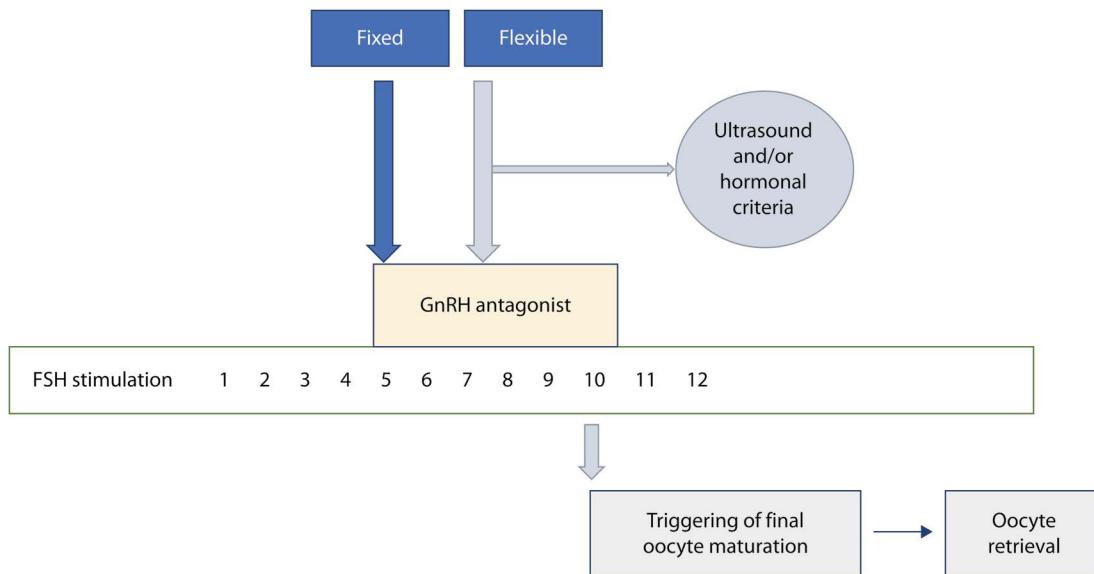


FIGURE 43.2 GnRH antagonist protocols. Fixed day 5 protocol: initiation of GnRH antagonist on day 5 of stimulation daily and continuation until the day of triggering final oocyte maturation. Flexible dose protocol: initiation of GnRH antagonist after certain endocrine and/or sonographic criteria indicating a risk for an LH rise are met and continuation until the day of triggering final oocyte maturation.

showed a decreased probability of live birth in patients pre-treated with OCP prior to initiation of gonadotrophin stimulation (OR 0.74, 95% CI 0.58–0.95) [39].

Moreover, OCP pre-treatment in GnRH antagonist cycles increases gonadotropin consumption (WMD: +360 IU, 95% CI +158 to +563 IU, $p < 0.01$) [39], which is a known advantage of GnRH antagonists over GnRH agonists [14].

Based on this data the ESHRE ovarian stimulation guideline strongly recommended against the use of combined oral contraceptive pill (COCP) pre-treatment (12–28 days) [19].

It should be noted, however, that it is still not clear whether the type of OCP pre-treatment regarding the oestrogen and progestogen components, the duration of OCP administration, or the starting day of gonadotrophin stimulation following OCP cessation [40] may alter the preceding recommendation [19].

Alternative ways that have been proposed to avoid weekend oocyte retrievals in GnRH antagonist cycles include delaying the day of starting gonadotropin stimulation from day 2 to day 3 of the cycle and/or postponing hCG administration by one day [41]. Conflicting results have been published regarding the effect of postponing hCC administration on the probability of pregnancy, which might be explained by differences in the criteria used for triggering final oocyte maturation, the population studied, and the number of patients analysed [42–45].

Delayed start GnRH antagonist protocol

In this protocol, ovarian stimulation is preceded by pre-treatment with oral oestradiol for 7–10 days in the late luteal phase, followed by seven days of GnRH antagonists. In this way, it is expected that suppression of endogenous FSH, prior to initiation of ovarian stimulation, will result in a more synchronous follicular growth. This protocol has been applied so far in poor responder patients and has been evaluated in two systematic reviews and meta-analyses [46, 47].

The latest one, published in 2021 [47], pooled the results of five RCTs, including 514 patients comparing the delayed start GnRH antagonist protocol with a flexible GnRH antagonist protocol. Delayed start GnRH antagonist protocol was associated with an increased probability of clinical pregnancy (RR = 2.30, 95% CI 1.38–3.82, $p = 0.001$). However, no data were available on the probability of ongoing pregnancy or live birth.

Gonadotrophin stimulation in a GnRH antagonist cycle

FSH starting dose

The optimal FSH starting dose is usually selected in IVF, based on the patient's body mass index, age, ovarian reserve (as assessed by antral follicle count and/or anti-Mullerian hormone) [48], and previous response to stimulation. However, efforts have also been made to determine it objectively [49, 50]. A starting dose of 150–200 IU is generally considered appropriate for a typical patient. Current evidence does not provide a clear justification for adjusting the standard dose of 150 IU in the case of poor or normal responders, especially as increased dose is generally associated with greater total FSH dose and therefore greater cost [51].

Initiation of gonadotropin stimulation

In GnRH antagonist cycles, FSH stimulation can start either on day 2 or day 3 of the cycle [37, 52], without affecting the chance of pregnancy [53]. A later initiation of FSH stimulation on day 5 is also possible in the so-called "mild stimulation protocols" [54], the target of which is increased safety and decreased drug consumption [55, 56]. The fact, however, that OHSS can nowadays be eliminated with the replacement of human chorionic gonadotrophin (hCG) by GnRH agonist [52] and a lower probability of pregnancy associated with mild stimulation have tempered the initial enthusiasm regarding these protocols [36].

Increasing the FSH dose at antagonist initiation

Increasing the FSH dose at GnRH antagonist initiation has so far been evaluated in two RCTs, which did not show a beneficial effect on the probability of clinical pregnancy (odds ratio for clinical pregnancy: 1.03, 95% CI 0.58–1.81) [57, 58].

Addition of LH to FSH

Addition of LH to FSH in GnRH antagonist cycles has been evaluated in numerous RCTs and summarized in several meta-analyses [59–62]. Based on the latest meta-analysis [61], LH addition does not appear to be beneficial in terms of pregnancy rate in GnRH antagonist cycles (Figure 43.3).

Long-acting FSH

Corifollitropin- α , produced by the fusion of recombinant FSH and the C-terminal peptide of the β -subunit of hCG, is characterized by a slower absorption and a longer half-life than daily recombinant FSH and has been licensed for use in GnRH antagonist cycles. Corifollitropin- α replaces seven days of standard recombinant FSH injections and achieves similar efficacy and safety [63], offering increased patient friendliness during ovarian stimulation for IVF [64].

The latest meta-analysis on the use of Corifollitropin- α in ovarian stimulation for IVF showed no difference in probability of live birth as compared to daily FSH (risk ratio [RR], 0.92; 95% confidence interval [CI], 0.80–1.05). Corifollitropin- α has also been used successfully in poor responder patients [65–67].

Endocrine associations in a GnRH antagonist cycle

Elevated serum progesterone, defined as progesterone >1.5 ng/mL, at initiation of stimulation in a spontaneous cycle following a natural luteal phase is a rather infrequent event in the general

population (~5% of patients). If, in those patients, initiation of stimulation is postponed for one or two days, progesterone levels will normalize in the majority of cases (80%). However, pregnancy rates in this group are expected to be significantly lower compared with patients with normal progesterone levels at initiation of stimulation [68, 69]. On the other hand, administration of GnRH antagonist for three consecutive days in patients with elevated progesterone on day 2 of the cycle has been shown to result in acceptable pregnancy rates compared to those achieved in patients with normal progesterone levels prior to gonadotropin initiation [70].

Elevated progesterone levels on the day of triggering final oocyte maturation have been associated with a significantly decreased probability of pregnancy (risk ratio: 0.76; 95% CI 0.60–0.97) [25]. If progesterone elevation occurs, it is worth considering freezing all embryos and performing the transfer in a subsequent cycle [71].

Low endogenous LH levels during ovarian stimulation with GnRH antagonists for pregnancy achievement should not raise concern and cannot serve as a rationale for LH addition to FSH. This was shown initially by Kolibianakis et al. in 2006 [72] and was subsequently confirmed in a large, individual patient data meta-analysis [73]. The odds ratios (95% CIs) for ongoing pregnancy for patients with LH levels less than the 25th centile and those with levels greater than the 25th centile on day 8 of stimulation as well as on the day of hCG administration were 0.96 (0.75–1.22) and 0.96 (0.76–1.21), respectively.

Triggering of final oocyte maturation in a GnRH antagonist cycle

Although the incidence of severe OHSS is significantly decreased in GnRH antagonist as compared to GnRH agonist cycles, OHSS can still occur (RR: 0.63, 95% CI 0.51–0.79) [18]. This is especially true in high-responder patients or those treated with excessive

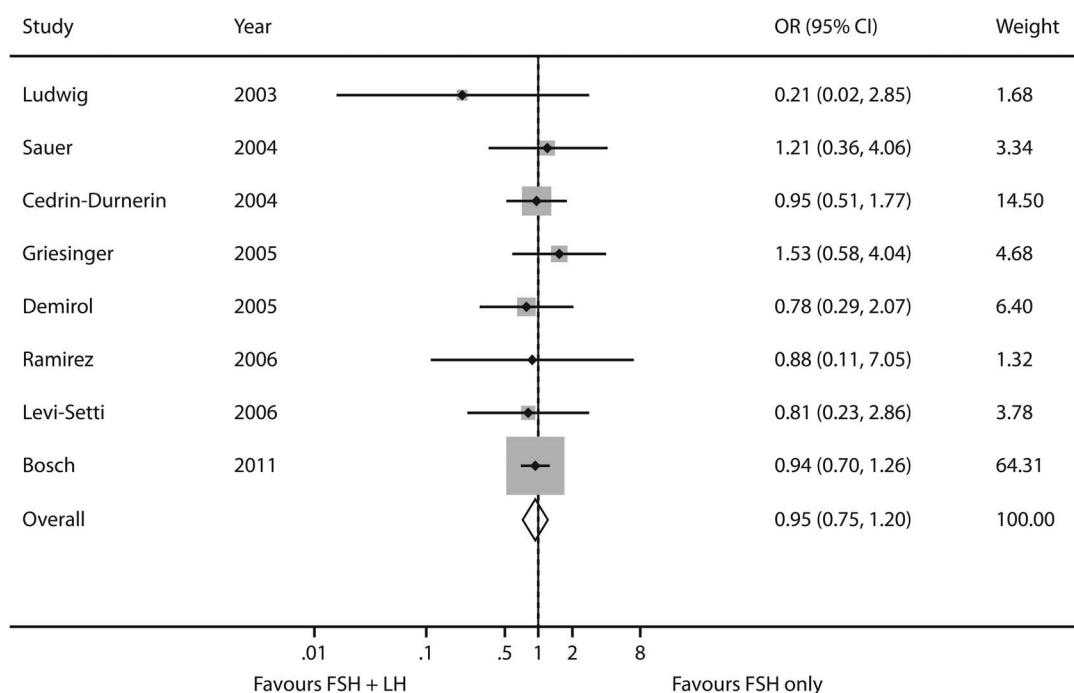


FIGURE 43.3 Addition of recombinant LH to recombinant FSH in gonadotropin-releasing hormone antagonist cycles. Abbreviations: CI, confidence interval; FSH, follicle-stimulating hormone; LH, luteinizing hormone; OR, odds ratio. (Based on [61].)

doses of gonadotropins, and is invariably associated with administration of hCG for triggering final oocyte maturation [74].

Thus, the unique option of replacing hCG with GnRH agonists in GnRH antagonist cycles represents one of the most important safety aspects of the antagonistic protocol [75]. This is due to the fact that GnRH agonists not only effectively induce final oocyte maturation [76], but at the same time eliminate the incidence of severe OHSS in an unsupported luteal phase [77]. This is the main reason that GnRH antagonists/FSH stimulation combined with GnRH agonist triggering today represents the standard mode of stimulation for oocyte donors and for the vast majority of freeze-all cycles [78, 79].

In patients using their own oocytes, however, if embryo transfer is performed in the same cycle under standard luteal-phase support, GnRH agonist triggering is associated with a significantly decreased probability of pregnancy [80, 81], due to alterations in the quality of the ensuing luteal phase. To manage this problem, three main approaches have been proposed: stimulation of corpora lutea [82–84]; administration of increased doses of sex steroids and freezing of all embryos and embryo transfer in subsequent cycles [85, 86].

The strategy of freezing all embryos after GnRH agonist triggering currently appears to be the safest approach [87] regarding the occurrence of severe OHSS, and, in addition, this approach maintains a high probability of pregnancy in subsequent freeze-thaw cycles [88].

Luteal support in GnRH antagonist cycles

Very low LH levels and endometrium abnormalities are present following oocyte retrieval in both GnRH agonist and antagonist cycles. These problems are associated with the supra-physiological sex steroid serum levels after gonadotropin stimulation, and they necessitate luteal phase support for pregnancy achievement [89].

Luteal-phase support is predominantly performed in both GnRH agonist and antagonist cycles by progesterone administration in the form of micronized vaginal progesterone [90] or intramuscular [91] or subcutaneous progesterone [92]. Initiation of progesterone for luteal phase support should occur in the window between the evening of the day of oocyte retrieval and day 3 post oocyte retrieval [19]. Progesterone for luteal phase support should be administered at least until the day of the pregnancy test [19].

Two RCTs, performed exclusively in GnRH antagonist cycles, did not suggest that addition of oestrogens to micronized progesterone increases the probability of ongoing pregnancy (risk ratio [RR]: 0.89, 95% CI 0.61–1.30) [93, 94].

New concepts in ovarian stimulation using GnRH antagonists

The introduction of GnRH antagonists has facilitated the development of new concepts, such as the modified natural cycle [95], mild IVF [96], and the initiation of antagonist in case of severe established OHSS [97–99], enhancing research and advancing progress in ovarian stimulation.

Moreover, from a patient perspective, the increased safety by eliminating severe OHSS, the improved patient friendliness by simplifying treatment with long-acting FSH and decreasing its duration, and finally the similar efficacy to GnRH agonists regarding the probability of live birth render GnRH antagonists the most attractive way to inhibit a premature LH rise in ovarian stimulation for IVF.

References

- Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet*. 1978;2(8085):366.
- Trounson AO, Leeton JF, Wood C, Webb J, Wood J. Pregnancies in humans by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. *Science*. 1981;212(4495):681–2.
- Janssens RM, Lambalk CB, Vermeiden JP, Schats R, Bernards JM, Rekers-Mombarg LT, et al. Dose-finding study of triptorelin acetate for prevention of a premature LH surge in IVF: A prospective, randomized, double-blind, placebo-controlled study. *Hum Reprod*. 2000;15(11):2333–40.
- Loumaye E. The control of endogenous secretion of LH by gonadotrophin-releasing hormone agonists during ovarian hyperstimulation for in-vitro fertilization and embryo transfer. *Hum Reprod*. 1990;5(4):357–76.
- Porter RN, Smith W, Craft IL, Abdulwahid NA, Jacobs HS. Induction of ovulation for in-vitro fertilisation using buserelin and gonadotropins. *Lancet*. 1984;2(8414):1284–5.
- Schally AV, Arimura A, Baba Y, Nair RM, Matsuo H, Redding TW, et al. Isolation and properties of the FSH and LH-releasing hormone. *Biochem Biophys Res Commun*. 1971;43(2):393–9.
- Guillemin R, Burgus R. The hormones of the hypothalamus. *Sci Am*. 1972;227(5):24–33.
- Hahn DW, McGuire JL, Vale WW, Rivier J. Reproductive/endocrine and anaphylactoid properties of an LHRH-antagonist, ORF 18260 [ac-DNAl(2), 4FDPhe2,D-Trp3,D-Arg6]-GnRH. *Life Sci*. 1985;37(6):505–14.
- Meldrum DR. Ovulation induction protocols. *Arch Pathol Lab Med*. 1992;116(4):406–9.
- Hughes EG, Fedorkow DM, Daya S, Sagle MA, Van de Koppel P, Collins JA. The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: A meta-analysis of randomized controlled trials. *Fertil Steril*. 1992;58(5):888–96.
- Marcus SF, Ledger WL. Efficacy and safety of long-acting GnRH agonists in in vitro fertilization and embryo transfer. *Hum Fertil (Camb)*. 2001;4(2):85–93.
- Duijkers IJ, Klipping C, Willemse WN, Krone D, Schneider E, Niebich G, et al. Single and multiple dose pharmacokinetics and pharmacodynamics of the gonadotrophin-releasing hormone antagonist cetrorelix in healthy female volunteers. *Hum Reprod*. 1998;13(9):2392–8.
- Mannaerts B, Gordon K. Embryo implantation and GnRH antagonists: GnRH antagonists do not activate the GnRH receptor. *Hum Reprod*. 2000;15(9):1882–3.
- Kolibianakis EM, Collins J, Tarlatzis BC, Devroey P, Diedrich K, Griesinger G. Among patients treated for IVF with gonadotrophins and GnRH analogues, is the probability of live birth dependent on the type of analogue used? A systematic review and meta-analysis. *Hum Reprod Update*. 2006;12(6):651–71.
- Al-Inany H, Aboulghar M. Gonadotrophin-releasing hormone antagonists for assisted conception. *Cochrane Database Syst Rev*. 2001(4):CD001750.
- Al-Inany HG, Abou-Setta AM, Aboulghar M. Gonadotrophin-releasing hormone antagonists for assisted conception. *Cochrane Database Syst Rev*. 2006(3):CD001750.
- Al-Inany HG, Youssef MA, Ayeleke RO, Brown J, Lam WS, Broekmans FJ. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev*. 2016;4:CD001750.
- Lambalk CB, Banga FR, Huirne JA, Toftager M, Pinborg A, Homburg R, et al. GnRH antagonist versus long agonist protocols in IVF: A systematic review and meta-analysis accounting for patient type. *Hum Reprod Update*. 2017;23(5):560–79.
- Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, Kolibianakis E, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI(dagger). *Hum Reprod Open*. 2020;2020(2):hoaa009.

20. Rabinovici J, Rothman P, Monroe SE, Nerenberg C, Jaffe RB. Endocrine effects and pharmacokinetic characteristics of a potent new gonadotropin-releasing hormone antagonist (Ganirelix) with minimal histamine-releasing properties: Studies in postmenopausal women. *J Clin Endocrinol Metab.* 1992;75(5):1220–5.
21. Klingmuller D, Schepke M, Enzweiler C, Bidlingmaier F. Hormonal responses to the new potent GnRH antagonist Cetrorelix. *Acta Endocrinol (Copenh).* 1993;128(1):15–8.
22. Diedrich K, Diedrich C, Santos E, Zoll C, al-Hasani S, Reissmann T, et al. Suppression of the endogenous luteinizing hormone surge by the gonadotrophin-releasing hormone antagonist Cetrorelix during ovarian stimulation. *Hum Reprod.* 1994;9(5):788–91.
23. Olivennes F, Fanchin R, Bouchard P, de Ziegler D, Taieb J, Selva J, et al. The single or dual administration of the gonadotropin-releasing hormone antagonist Cetrorelix in an in vitro fertilization-embryo transfer program. *Fertil Steril.* 1994;62(3):468–76.
24. Albano C, Smitz J, Camus M, Riethmüller-Winzen H, Van Steirteghem A, Devroey P. Comparison of different doses of gonadotropin-releasing hormone antagonist Cetrorelix during controlled ovarian hyperstimulation. *Fertil Steril.* 1997;67(5):917–22.
25. The ganirelix dose-finding study group. A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist ganirelix (Org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon). *Hum Reprod.* 1998;13(11):3023–31.
26. Borm G, Mannaerts B, The European Orgalutran Study Group. Treatment with the gonadotrophin-releasing hormone antagonist ganirelix in women undergoing ovarian stimulation with recombinant follicle stimulating hormone is effective, safe and convenient: Results of a controlled, randomized, multicentre trial. *Hum Reprod.* 2000;15(7):1490–8.
27. Devroey P, Boostanfar R, Koper NP, Mannaerts BM, Ijzerman-Boon PC, Fauser BC, et al. A double-blind, non-inferiority RCT comparing corifollitropin alfa and recombinant FSH during the first seven days of ovarian stimulation using a GnRH antagonist protocol. *Hum Reprod.* 2009;24(12):3063–72.
28. Kolibianakis EM, Venetis CA, Kalogeropoulou L, Papanikolaou E, Tarlatzis BC. Fixed versus flexible gonadotropin-releasing hormone antagonist administration in in vitro fertilization: A randomized controlled trial. *Fertil Steril.* 2011;95(2):558–62.
29. Kolibianaki E, Kolibianakis E. The GnRH antagonist protocol. in press. 2021.
30. Escudero E, Bosch E, Crespo J, Simon C, Remohi J, Pellicer A. Comparison of two different starting multiple dose gonadotropin-releasing hormone antagonist protocols in a selected group of in vitro fertilization-embryo transfer patients. *Fertil Steril.* 2004;81(3):562–6.
31. Ludwig M, Katalinic A, Banz C, Schroder AK, Loning M, Weiss JM, et al. Tailoring the GnRH antagonist cetrorelix acetate to individual patients' needs in ovarian stimulation for IVF: Results of a prospective, randomized study. *Hum Reprod.* 2002;17(11):2842–5.
32. Mochtar MH, Dutch Ganirelix Study G. The effect of an individualized GnRH antagonist protocol on folliculogenesis in IVF/ICSI. *Hum Reprod.* 2004;19(8):1713–8.
33. Depalo R, Trerotoli P, Chincoli A, Vacca MP, Lamanna G, Cincinelli E. Endogenous luteinizing hormone concentration and IVF outcome during ovarian stimulation in fixed versus flexible GnRH antagonist protocols: An RCT. *Int J Reprod Biomed.* 2018;16(3):175–82.
34. Hosseini RB, Behrouzi LT, ShahrokhTehrani E, Davari TF. Fixed versus flexible gonadotropin releasing hormone antagonist protocol in controlled ovarian stimulation for invitro fertilization in women with polycystic ovary syndrome. *J Family Reprod Health.* 2015;9(3):141–6.
35. Luo X, Pei L, Li F, Li C, Huang G, Ye H. Fixed versus flexible antagonist protocol in women with predicted high ovarian response except PCOS: A randomized controlled trial. *BMC Pregnancy Childbirth.* 2021;21(1):348.
36. Fauser BC, Alper MM, Ledger W, Schoolcraft WB, Zandvliet A, Mannaerts BM, et al. Pharmacokinetics and follicular dynamics of corifollitropin alfa versus recombinant FSH during ovarian stimulation for IVF. *Reprod Biomed Online.* 2010;21(5):593–601.
37. European Middle East Orgalutran Study G. Comparable clinical outcome using the GnRH antagonist ganirelix or a long protocol of the GnRH agonist triptorelin for the prevention of premature LH surges in women undergoing ovarian stimulation. *Hum Reprod.* 2001;16(4):644–51.
38. Fischl F, Huber JC, Obruca A. Zeitliche Optimierung der kontrollierten Hyperstimulation (KOH) in Kombination mit GnRH-Antagonisten und Ovulationshemmer in einem IVF-Programm. *J Fertil Reprod.* 2001. 2001;11:50–1.
39. Farquhar C, Rombauts L, Kremer JA, Lethaby A, Ayeleke RO. Oral Contraceptive pill, progestogen or oestrogen pre-treatment for ovarian stimulation protocols for women undergoing assisted reproductive techniques. *Cochrane Database Syst Rev.* 2017;5:CD006109.
40. Cedrin-Durnerin I, Bstandig B, Parneix I, Bied-Damon V, Avril C, Decanter C, et al. Effects of oral contraceptive, synthetic progestogen or natural estrogen pre-treatments on the hormonal profile and the antral follicle cohort before GnRH antagonist protocol. *Hum Reprod.* 2007;22(1):109–16.
41. Gordon KLM, Ledger W, Kolibianakis EM, Ijzerman-Boon PC. Reducing the incidence of weekend oocyte retrievals in a rFSH/GnRH antagonist protocol by optimizing the start day of rFSH and delaying human chorionic gonadotropin (hCG) by 1 day. *Fertil Steril.* 2011;95(A double-blind): S16–S.
42. Kolibianakis EM, Albano C, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Prolongation of the follicular phase in in vitro fertilization results in a lower ongoing pregnancy rate in cycles stimulated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonists. *Fertil Steril.* 2004;82(1):102–7.
43. Kyrou D, Kolibianakis EM, Fatemi HM, Tarlatzis BC, Tournaye H, Devroey P. Is earlier administration of human chorionic gonadotropin (hCG) associated with the probability of pregnancy in cycles stimulated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone (GnRH) antagonists? A prospective randomized trial. *Fertil Steril.* 2011;96(5):1112–5.
44. Mochtar MH, Custers IM, Koks CA, Bernardus RE, Verhoeve HR, Mol BW, et al. Timing oocyte collection in GnRH agonists down-regulated IVF and ICSI cycles: A randomized clinical trial. *Hum Reprod.* 2011;26(5):1091–6.
45. Morley L, Tang T, Yasmin E, Hamzeh R, Rutherford AJ, Balen AH. Timing of human chorionic gonadotrophin (hCG) hormone administration in IVF protocols using GnRH antagonists: A randomized controlled trial. *Hum Fertil (Camb).* 2012;15(3):134–9.
46. Cozzolino M, Fransasiak J, Andrisani A, Ambrosini G, Vitagliano A. “Delayed start” gonadotropin-releasing hormone antagonist protocol in Bologna poor-responders: A systematic review and meta-analysis of randomized controlled trials. *Eur J Obstet Gynecol Reprod Biol.* 2020;244:154–62.
47. Yang S, Liu N, Li Y, Zhang L, Yue R. Efficacy of the delayed start antagonist protocol for controlled ovarian stimulation in Bologna poor ovarian responders: A systematic review and meta-analysis. *Arch Gynecol Obstet.* 2021;303(2):347–62.
48. Broer SL, Dolleman M, Opmeer BC, Fauser BC, Mol BW, Broekmans FJ. AMH and AFC as predictors of excessive response in controlled ovarian hyperstimulation: A meta-analysis. *Hum Reprod Update.* 2011;17(1):46–54.

49. Olivennes F, Howles CM, Borini A, Germond M, Trew G, Wiklund M, et al. Individualizing FSH dose for assisted reproduction using a novel algorithm: The CONSORT study. *Reprod Biomed Online.* 2009;18(2):195–204.
50. Popovic-Todorovic B, Loft A, Bredkjaer HE, Bangsboll S, Nielsen IK, Andersen AN. A prospective randomized clinical trial comparing an individual dose of recombinant FSH based on predictive factors versus a 'standard' dose of 150 IU/day in 'standard' patients undergoing IVF/ICSI treatment. *Hum Reprod.* 2003;18(11):2275–82.
51. Lensen SF, Wilkinson J, Leijdekkers JA, La Marca A, Mol BWJ, Marjoribanks J, et al. Individualised gonadotropin dose selection using markers of ovarian reserve for women undergoing in vitro fertilisation plus intracytoplasmic sperm injection (IVF/ICSI). *Cochrane Database Syst Rev.* 2018;2:CD012693.
52. Albano C, Felberbaum RE, Smitz J, Riethmuller-Winzen H, Engel J, Diedrich K, et al. Ovarian stimulation with HMG: Results of a prospective randomized phase III European study comparing the luteinizing hormone-releasing hormone (LHRH)-antagonist cetrorelix and the LHRH-agonist buserelin. European Cetrorelix Study Group. *Hum Reprod.* 2000;15(3):526–31.
53. Levy MJ, Ledger W, Kolibianakis EM, Ijzerman-Boon PC, Gordon K. Is it possible to reduce the incidence of weekend oocyte retrievals in GnRH antagonist protocols? *Reprod Biomed Online.* 2013;26(1):50–8.
54. Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. *J Clin Endocrinol Metab.* 2003;88(1):166–73.
55. Verberg MF, Eijkemans MJ, Macklon NS, Heijnen EM, Baart EB, Hohmann FP, et al. The clinical significance of the retrieval of a low number of oocytes following mild ovarian stimulation for IVF: A meta-analysis. *Hum Reprod Update.* 2009;15(1):5–12.
56. Verberg MF, Macklon NS, Nargund G, Frydman R, Devroey P, Broekmans FJ, et al. Mild ovarian stimulation for IVF. *Hum Reprod Update.* 2009;15(1):13–29.
57. Aboulghar MA, Mansour RT, Serour GI, Al-Inany HG, Amin YM, Aboulghar MM. Increasing the dose of human menopausal gonadotrophins on day of GnRH antagonist administration: Randomized controlled trial. *Reprod Biomed Online.* 2004;8(5):524–7.
58. Propst AM, Bates GW, Robinson RD, Arthur NJ, Martin JE, Neal GS. A randomized controlled trial of increasing recombinant follicle-stimulating hormone after initiating a gonadotropin-releasing hormone antagonist for in vitro fertilization-embryo transfer. *Fertil Steril.* 2006;86(1):58–63.
59. Baruffi RL, Mauri AL, Petersen CG, Felipe V, Martins AM, Cornicelli J, et al. Recombinant LH supplementation to recombinant FSH during induced ovarian stimulation in the GnRH-antagonist protocol: A meta-analysis. *Reprod Biomed Online.* 2007;14(1):14–25.
60. Kolibianakis EM, Kalogeropoulou L, Griesinger G, Papanikolaou EG, Papadimas J, Bontis J, et al. Among patients treated with FSH and GnRH analogues for in vitro fertilization, is the addition of recombinant LH associated with the probability of live birth? A systematic review and meta-analysis. *Hum Reprod Update.* 2007;13(5):445–52.
61. Lehert P, Kolibianakis EM, Venetis CA, Schertz J, Saunders H, Arriagada P, et al. Recombinant human follicle-stimulating hormone (r-hFSH) plus recombinant luteinizing hormone versus r-hFSH alone for ovarian stimulation during assisted reproductive technology: Systematic review and meta-analysis. *Reprod Biol Endocrinol.* 2014;12:17.
62. Mochtar MH, Van der V, Ziech M, van Wely M. Recombinant luteinizing hormone (rLH) for controlled ovarian hyperstimulation in assisted reproductive cycles. *Cochrane Database Syst Rev.* 2007;(2): CD005070.
63. Griesinger G, Boostanfar R, Gordon K, Gates D, McCrary Sisk C, Stegmann BJ. Corifollitropin alfa versus recombinant follicle-stimulating hormone: An individual patient data meta-analysis. *Reprod Biomed Online.* 2016;33(1):56–60.
64. Rombauts L, Talmor A. Corifollitropin alfa for female infertility. *Expert Opin Biol Ther.* 2012;12(1):107–12.
65. Kolibianakis EM, Venetis CA, Bosdou JK, Zepiridis L, Chatzimeletiou K, Makedos A, et al. Corifollitropin alfa compared with follitropin beta in poor responders undergoing ICSI: A randomized controlled trial. *Hum Reprod.* 2015;30(2):432–40.
66. Rinaldi L, Selman H. Profile of follitropin alpha/lutropin alpha combination for the stimulation of follicular development in women with severe luteinizing hormone and follicle-stimulating hormone deficiency. *Int J Womens Health.* 2016;8:169–79.
67. Taranger R, Martinez-Cuenca S, Ferreros I, Rubio JM, Fernandez-Colom PJ, Martinez-Triguero ML, et al. Ovarian stimulation with corifollitropin alfa followed by hp-hMG compared to hp-hMG in patients at risk of poor ovarian response undergoing ICSI: A randomized controlled trial. *Eur J Obstet Gynecol Reprod Biol.* 2018;231:192–7.
68. Hamidine O, Macklon NS, Eijkemans MJ, Laven JS, Cohlen BJ, Verhoeff A, et al. Elevated early follicular progesterone levels and in vitro fertilization outcomes: A prospective intervention study and meta-analysis. *Fertil Steril.* 2014;102(2):448–54 e1.
69. Kolibianakis EM, Zikopoulos K, Smitz J, Camus M, Tournaye H, Steirteghem Van AC, et al. Elevated progesterone at initiation of stimulation is associated with a lower ongoing pregnancy rate after IVF using GnRH antagonists. *Hum Reprod.* 2004;19(7):1525–9.
70. Blockeel C, Baumgarten M, De Vos M, Verheyen G, Devroey P. Administration of GnRH antagonists in case of elevated progesterone at initiation of the cycle: A prospective cohort study. *Curr Pharm Biotechnol.* 2011;12(3):423–8.
71. Venetis CA, Kolibianakis EM, Bosdou JK, Tarlatzis BC. Progesterone elevation and probability of pregnancy after IVF: A systematic review and meta-analysis of over 60000 cycles. *Hum Reprod Update.* 2013;19(5):433–57.
72. Kolibianakis EM, Collins J, Tarlatzis B, Papanikolaou E, Devroey P. Are endogenous LH levels during ovarian stimulation for IVF using GnRH analogues associated with the probability of ongoing pregnancy? A systematic review. *Hum Reprod Update.* 2006;12(1):3–12.
73. Griesinger G, Shapiro DB, Kolibianakis EM, Witjes H, Mannaerts BM. No association between endogenous LH and pregnancy in a GnRH antagonist protocol: Part II, recombinant FSH. *Reprod Biomed Online.* 2011;23(4):457–65.
74. Lainas GT, Lainas TG, Sfontouris IA, Chatzimeletiou K, Venetis CA, Bosdou JK, et al. Is oocyte maturation rate associated with triptorelin dose used for triggering final oocyte maturation in patients at high risk for severe ovarian hyperstimulation syndrome? *Hum Reprod.* 2019;34(9):1770–7.
75. Kolibianakis EM, Venetis CA, Bontis J, Tarlatzis BC. Significantly lower pregnancy rates in the presence of progesterone elevation in patients treated with GnRH antagonists and gonadotrophins: A systematic review and meta-analysis. *Curr Pharm Biotechnol.* 2012;13(3):464–70.
76. Griesinger G, Diedrich K, Devroey P, Kolibianakis EM. GnRH agonist for triggering final oocyte maturation in the GnRH antagonist ovarian hyperstimulation protocol: A systematic review and meta-analysis. *Hum Reprod Update.* 2006;12(2):159–68.
77. Tarlatzis B, BJ, Kolibianakis S. Elimination of OHSS by GnRH agonist and freezing embryos. In Rizk B, and Gerris J, (Eds.), *Complications and Outcomes of Assisted Reproduction* (pp. 141–148). Cambridge: Cambridge University Press, 2017. doi: 10.1017/9781107295391.015.
78. Bodri D, Sunkara SK, Coomarasamy A. Gonadotropin-releasing hormone agonists versus antagonists for controlled ovarian hyperstimulation in oocyte donors: A systematic review and meta-analysis. *Fertil Steril.* 2011;95(1):164–9.

79. Mizrahi Y, Horowitz E, Farhi J, Raziel A, Weissman A. Ovarian stimulation for freeze-all IVF cycles: A systematic review. *Hum Reprod Update*. 2020;26(1):118–35.
80. Humaidan P, Bredkjaer HE, Bungum L, Bungum M, Grondahl ML, Westergaard L, et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomized study. *Hum Reprod*. 2005;20(5):1213–20.
81. Kolibianakis EM, Schultze-Mosgau A, Schroer A, van Steirteghem A, Devroey P, Diedrich K, et al. A lower ongoing pregnancy rate can be expected when GnRH agonist is used for triggering final oocyte maturation instead of HCG in patients undergoing IVF with GnRH antagonists. *Hum Reprod*. 2005;20(10):2887–92.
82. Humaidan P, Polyzos NP, Alsberg B, Erb K, Mikkelsen AL, Elbaek HO, et al. GnRH_a trigger and individualized luteal phase hCG support according to ovarian response to stimulation: Two prospective randomized controlled multi-centre studies in IVF patients. *Hum Reprod*. 2013;28(9):2511–21.
83. Papanikolaou EG, Verpoest W, Fatemi H, Tarlatzis B, Devroey P, Tournaye H. A novel method of luteal supplementation with recombinant luteinizing hormone when a gonadotropin-releasing hormone agonist is used instead of human chorionic gonadotropin for ovulation triggering: A randomized prospective proof of concept study. *Fertil Steril*. 2011;95(3):1174–7.
84. Pirard C, Donnez J, Loumiae E. GnRH agonist as novel luteal support: Results of a randomized, parallel group, feasibility study using intranasal administration of buserelin. *Hum Reprod*. 2005;20(7):1798–804.
85. Devroey P, Polyzos NP, Blockeel C. An OHSS-Free Clinic by segmentation of IVF treatment. *Hum Reprod*. 2011;26(10):2593–7.
86. Griesinger G, Kolibianakis EM, Papanikolaou EG, Diedrich K, Van Steirteghem A, Devroey P, et al. Triggering of final oocyte maturation with gonadotropin-releasing hormone agonist or human chorionic gonadotropin. Live birth after frozen-thawed embryo replacement cycles. *Fertil Steril*. 2007;88(3):616–21.
87. Ioannidou PG, Bosdou JK, Lainas GT, Lainas TG, Grimbizis GF, Kolibianakis EM. How frequent is severe ovarian hyperstimulation syndrome after GnRH agonist triggering in high-risk women? A systematic review and meta-analysis. *Reprod Biomed Online*. 2021;42(3):635–50.
88. Bosdou JK, Venetis CA, Tarlatzis BC, Grimbizis GF, Kolibianakis EM. Higher probability of live-birth in high, but not normal, responders after first frozen-embryo transfer in A freeze-only cycle strategy compared to fresh-embryo transfer: A meta-analysis. *Hum Reprod*. 2019;34(3):491–505.
89. Beckers NG, Macklon NS, Eijkemans MJ, Ludwig M, Felberbaum RE, Diedrich K, et al. Nonsupplemented luteal phase characteristics after the administration of recombinant human chorionic gonadotropin, recombinant luteinizing hormone, or gonadotropin-releasing hormone (GnRH) agonist to induce final oocyte maturation in in vitro fertilization patients after ovarian stimulation with recombinant follicle-stimulating hormone and GnRH antagonist cotreatment. *J Clin Endocrinol Metab*. 2003;88(9):4186–92.
90. Feinberg EC, Beltsos AN, Nicolaou E, Marut EL, Uhler ML. Endometrin as luteal phase support in assisted reproduction. *Fertil Steril*. 2013;99(1):174–8 e1.
91. Yanushpolsky E, Hurwitz S, Greenberg L, Racowsky C, Hornstein M. Crinone vaginal gel is equally effective and better tolerated than intramuscular progesterone for luteal phase support in in vitro fertilization-embryo transfer cycles: A prospective randomized study. *Fertil Steril*. 2010;94(7):2596–9.
92. Baker VL, Jones CA, Doody K, Foulk R, Yee B, Adamson GD, et al. A randomized, controlled trial comparing the efficacy and safety of aqueous subcutaneous progesterone with vaginal progesterone for luteal phase support of in vitro fertilization. *Hum Reprod*. 2014;29(10):2212–20.
93. Ceyhan ST, Basaran M, Kemal Duru N, Yilmaz A, Goktolga U, Baser I. Use of luteal estrogen supplementation in normal responder patients treated with fixed multidose GnRH antagonist: A prospective randomized controlled study. *Fertil Steril*. 2008;89(6):1827–30.
94. Fatemi HM, Kolibianakis EM, Camus M, Tournaye H, Donoso P, Papanikolaou E, et al. Addition of estradiol to progesterone for luteal supplementation in patients stimulated with GnRH antagonist/rFSH for IVF: A randomized controlled trial. *Hum Reprod*. 2006;21(10):2628–32.
95. Lainas TG, Sfontouris IA, Venetis CA, Lainas GT, Zorzosilis IZ, Tarlatzis BC, et al. Live birth rates after modified natural cycle compared with high-dose FSH stimulation using GnRH antagonists in poor responders. *Hum Reprod*. 2015;30(10):2321–30.
96. Fauser BC, Nargund G, Andersen AN, Norman R, Tarlatzis B, Boivin J, et al. Mild ovarian stimulation for IVF: 10 years later. *Hum Reprod*. 2010;25(11):2678–84.
97. Lainas TG, Sfontouris IA, Zorzosilis IZ, Petsas GK, Lainas GT, Alexopoulou E, et al. Live births after management of severe OHSS by GnRH antagonist administration in the luteal phase. *Reprod Biomed Online*. 2009;19(6):789–95.
98. Lainas TG, Sfontouris IA, Zorzosilis IZ, Petsas GK, Lainas GT, Iliadis GS, et al. Management of severe OHSS using GnRH antagonist and blastocyst cryopreservation in PCOS patients treated with long protocol. *Reprod Biomed Online*. 2009;18(1):15–20.
99. Lainas TG, Sfontouris IA, Zorzosilis IZ, Petsas GK, Lainas GT, Kolibianakis EM. Management of severe early ovarian hyperstimulation syndrome by re-initiation of GnRH antagonist. *Reprod Biomed Online*. 2007;15(4):408–12.

44

GnRH AGONIST TRIGGERING

Katherine Koniares, Leah Kaye, Claudio Benadiva, and Lawrence Engmann

Overview

The use of gonadotropin releasing hormone agonist (GnRHa) has been advocated as a substitute to human chorionic gonadotropin (hCG) for the induction of oocyte maturation and prevention of ovarian hyperstimulation syndrome (OHSS) during IVF cycles since the late 1980s to early 90s [1–8]. However, the subsequent widespread use of GnRHa for pituitary downregulation during controlled ovarian stimulation limited its use as an option for triggering oocyte maturation.

After GnRH antagonists were introduced for prevention of the LH surge during controlled ovarian stimulation in the late 1990s, GnRHa could then be used again for the induction of oocyte maturation [9–11]. GnRH antagonist blocks the GnRH receptors on the pituitary by competitive inhibition [12]. Administration of GnRHa will then displace the antagonist on the receptors and activate them to promote a release of gonadotropins stored in the anterior pituitary [13].

More than 20 years after the first publication regarding the use of GnRHa trigger after GnRH antagonist pre-treatment during IVF [11], there are still several questions regarding its effectiveness in inducing oocyte maturation and the ideal luteal phase supplementation protocol (Table 44.1). Early clinical experiences in the mid-2000s were published using GnRHa for trigger during antagonist stimulation cycles [11]. Unfortunately, early studies reported high early pregnancy loss rates and low clinical pregnancy rates [14, 15]. Additional studies have subsequently been published in an effort to understand the underlying causes of the suboptimal pregnancy rates and to improve the clinical efficacy of the GnRHa trigger. The culmination of current literature now suggests that the luteolytic properties of GnRHa are effective in preventing OHSS but are also likely the cause of low pregnancy rates when standard luteal support is used. By optimizing the luteal phase profile for fresh transfer after GnRHa trigger, pregnancy rates can be comparable to those of the hCG trigger while reducing or eliminating the risks of OHSS [16–29].

Indications

In the current setting of ART there are clinical situations in which a GnRHa trigger should be considered first line due to

TABLE 44.1 Controversies Surrounding Use of GnRHa Trigger

1. What is the ideal dose of GnRHa trigger?
2. Is it effective in inducing oocyte maturation?
3. Are there any post-trigger serum LH or P levels that will predict trigger failure?
4. Does it eliminate the risk of OHSS?
5. Should fresh embryo transfer be performed or should all oocytes/embryos be frozen after GnRHa trigger?
6. What is the ideal luteal-phase supplementation regimen?

TABLE 44.2 Indications for GnRHa Trigger

High risk for OHSS development
Oocyte donors
Elective cryopreservation of oocytes or embryos
<ul style="list-style-type: none">• Fertility preservation for medical reasons, e.g. cancer• Fertility preservation for social reasons• Trophectoderm biopsy for PGT• Premature serum progesterone rise prior to induction of oocyte maturation

the benefits of safety and comfort for the patient (Table 44.2). In particular, any patient who does not plan to have a fresh embryo transfer may be an ideal candidate for GnRHa trigger, including patients treated for fertility preservation, trophectoderm biopsy for pre-implantation genetic testing (PGT), or prematurely elevated progesterone prior to trigger [30–32]. Young, healthy women undergoing oocyte donation are also ideal candidates for GnRHa trigger. Moreover, any woman at risk of developing OHSS is an ideal candidate for GnRHa trigger with modified luteal phase support or subsequent elective cryopreservation of oocytes or embryos.

Some patients are not well suited for use of a GnRHa trigger as it relies on the ability to mount an endogenous surge of gonadotropins. As a result, patients with hypothalamic dysfunction are not ideal candidates for GnRHa trigger for oocyte maturation. Moreover, women who have had long-term suppression of the hypothalamus and pituitary may have a failed or suboptimal response because they may not be able to mount an optimal LH surge after GnRHa trigger [33].

Physiology

Natural versus GnRHa-induced mid-cycle surge

A single bolus of GnRHa will interact with the GnRH receptors and cause the endogenous release, or “flare,” of gonadotropins from the anterior pituitary. The resultant surge of LH and FSH resembles the natural mid-cycle surge of gonadotropins seen shortly before ovulation, and thus a bolus of GnRHa can “trigger” ovulation [34]. While the role of the FSH surge is not completely elucidated in humans, there are animal and human cell studies suggesting that FSH plays a role in oocyte maturation and resumption of meiosis [35, 36], function of the oocyte–cumulus complex and facilitation of its detachment from the follicle wall [37], and generation of LH receptors on granulosa cells [38]. Thus there may be advantages to an ovulation trigger that results in a surge of both LH and FSH.

A natural ovulatory surge consists traditionally of three phases: abrupt onset (14 hours), LH peak/plateau (14 hours), and gradual descent to baseline (20 hours), lasting a mean duration of 48 hours (Figure 44.1) [39]. In contrast, the surge after a GnRHa

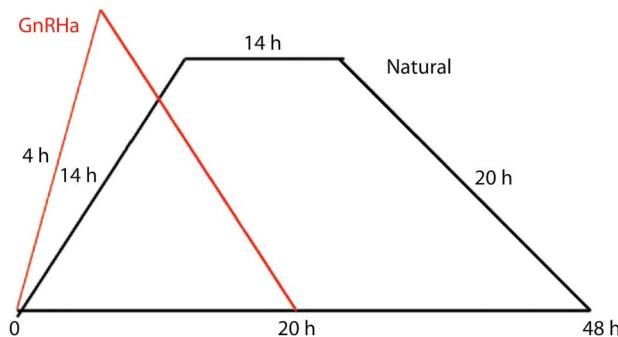


FIGURE 44.1 Luteinizing hormone surge in a natural cycle and after GnRH agonist trigger. (From [141], with permission.)

trigger occurs in two phases, rapid ascent and moderate descent, lasting 24–36 hours [3]. An early study by Itskovitz et al. showed that GnRHa causes LH to rise over four hours and FSH to rise over 12 hours, with significant elevation lasting 24 hours before a return of LH to baseline levels [3, 20]. The relatively short duration of the LH surge is capable of inducing oocyte maturation and ovulation but may result in defective formation of the corpus luteum [40].

Follicular fluid and granulosa/luteal cells after GnRHa trigger

Differences in follicular fluid dynamics between GnRHa and hCG trigger may explain potential differences in the induction of oocyte maturation, prevention of OHSS, and pregnancy rates. Follicular fluid after GnRHa trigger is noted to have significantly higher levels of LH and FSH than that after hCG trigger due to the combined surge of both gonadotropins [41]. Progesterone levels are reduced by 25%, attributed to a lack of LH stimulus on luteal cells in the GnRHa trigger group [41, 42]. Levels of oestrogen, inhibin-A, and inhibin-B have been shown to be similar after both triggers [42]. These differences in follicular fluid dynamics may represent a larger difference between the signal required for oocyte maturation versus the signal needed for ovulation; although they are typically two closely related events, they may require slightly different signals [42]. The follicular fluid studies reflect the similarity between the GnRHa surge and the natural mid-cycle surge, with an endogenous surge of LH and FSH and resultant oocyte maturity, but also how pregnancy rates may be affected by decreased luteal phase progesterone seen in both the follicular fluid and in the circulation.

Amphiregulin and other members of the epidermal growth factor (EGF)-like family rapidly increase in follicular fluid in response to LH/hCG and are felt to play a role in oocyte maturation by mediating the LH effects within the follicle [43]. Levels of amphiregulin in follicular fluid after GnRHa trigger are much lower than in follicles triggered with hCG and approach the level of a natural cycle [44]. Vascular endothelial growth factor (VEGF) is also noted to be significantly decreased in follicular fluid after GnRHa trigger, and expression for VEGF mRNA in the granulosa cells is decreased when compared to hCG trigger [45]. VEGF is one of the key vasoactive substances and works in part by modulating endothelial cell permeability and hyperpermeability via the cell adhesion molecule VE-cadherin within the ovarian cells [46]. The significant decrease in VEGF and vascular permeability after GnRHa trigger plays a major role in the prevention of OHSS [45]. Closely related is angiopoietin-2 (Ang-2), which causes

vascular destabilization and may work synergistically with VEGF to promote the leakage of fluid into the third space that occurs in OHSS. Cerrillo et al. found a decrease in Ang-2 levels in follicular fluid when using a GnRHa trigger but only to non-significant levels [45, 47]. It has also been shown that GnRHa induces a direct effect on granulosa cell expression of an antiangiogenic factor, pigment epithelium derived factor (PEDF), and thereby increases the PEDF to VEGF ratio and creates a more antiangiogenic environment which may result in impairment of corpus lutea function and hence the onset of OHSS [48].

Although rapid luteolysis occurs after GnRHa trigger, granulosa/luteal cells maintain similar functionality and viability within the first two days after trigger when compared with hCG trigger [49]. Engmann et al. analysed luteal cells collected at oocyte retrieval and noted no significant difference in the proportion of apoptotic cells [49]. Finally, the authors showed that luteal cells after both triggers remained responsive, and when exposed to hCG *in vitro* were able to increase progesterone production [49].

Administration

A number of different GnRH agonists are available for subcutaneous injection, including triptorelin, buserelin, leuprorelin, and nafarelin. Buserelin and nafarelin are available as an intranasal spray. All must be used for induction of oocyte maturation in IVF stimulation protocols without pituitary suppression or those that utilize a GnRH antagonist or progesterone primed ovarian stimulation (PPOS) for suppression of the LH surge.

Very few studies have been performed to determine the optimal trigger dose that will effectively induce oocyte maturation and prevent OHSS by minimizing luteolysis. Different doses of subcutaneous leuprorelin have been used in the literature and range from 0.5 mg to 4 mg [19, 20, 29, 31, 50–52]. Although some studies have used a higher dose of leuprorelin, 4 mg [29], and others have used two doses 12 hours apart [53], a single dose of 1 mg is effective in inducing optimal mature oocyte yield [54]. The dose of triptorelin has consistently been 0.2 mg in the literature [11, 15, 16, 20, 55, 56]. However, a randomized dose-finding study of 0.2, 0.3, and 0.4 mg of triptorelin in oocyte donors showed similar rates of mature oocytes and top-quality embryos regardless of dose [57]. More recently, a retrospective cohort study found similar rates of oocyte maturation with a triptorelin dose of 0.1 mg compared with higher doses of 0.2 and 0.4 mg [58]. Additionally, a prospective cohort study found a lower number of metaphase II (MII) oocytes in patients with a body mass index $\geq 25 \text{ kg/m}^2$ compared to patients with a body mass index $< 25 \text{ kg/m}^2$ who had received triptorelin 0.2 mg [59]. Given the ever-increasing rates of obesity, further investigation into the role of body mass index and dosing of GnRHa is needed. Although different doses have been used for intranasal buserelin, Buckett et al. showed that a dose of 50 micrograms is the most effective minimal dose to induce an endogenous surge consistently [60]. Given the overall equivalent findings, availability and cost should be considered in choosing the type and dose of GnRHa to use for trigger of oocyte maturation. As there may be differences in the endocrine profiles of the luteal phase due to differences in trigger dose, further fine-tuning of the trigger dose could enhance the function of the corpora lutea and overall outcomes.

There has been little research investigating the timing of GnRHa administration with regard to the previous GnRH antagonist dose. In order for GnRHa to trigger an LH surge, it must

displace GnRH antagonist from its receptors in the pituitary. A study by Horowitz et al. found a similar number of oocytes retrieved, MII oocytes, and fertilization rate among patients who administered GnRHa trigger <2.5 hours to >7 hours following their last dose of GnRH antagonist [61]. In order to optimize outcomes, further research is needed to delineate the ideal timing of GnRHa trigger following administration of the final GnRH antagonist dose.

Oocyte yield after GnRHa trigger

The GnRHa trigger has been shown to be as effective as hCG trigger with respect to oocyte yield and maturity in both autologous and donor cycles (Table 44.3). Some studies suggest that a GnRHa trigger may result in more mature oocytes [14, 24, 32, 62–66], though other studies did not [17, 19, 27, 28, 67–70]. Humaidan et al. found in a randomized trial of 122 patients that GnRHa trigger resulted in 16% more MII oocytes than hCG ($p < 0.02$) [14]. A later study by the same group resulted in 14% more MII oocytes and 11% more embryos suitable for transfer after GnRHa trigger compared to hCG [44]. Time-lapse analysis demonstrated that embryos originating from GnRHa-triggered cycles cleave earlier than embryos derived from hCG-triggered cycles [71]. Variations in early embryo development may stem from exposure to a different hormone milieu at the time of oocyte maturation, with more high-quality embryos resulting from GnRHa- compared with hCG-triggered cycles.

In a study including 508 cycles triggered with only GnRHa, Kummer et al. found that there were no clear serum predictors for oocyte yield, but post-trigger LH and progesterone strongly correlated with total oocytes and mature oocytes retrieved [54]. The authors showed that all cases of empty follicle syndrome (EFS) had an LH <15 IU/L and progesterone ≤ 3.5 ng/mL measured 8–12 hours after trigger. The probability of EFS occurring with a post-trigger LH less than 15 IU/L was 18.8%. A similar study evaluating post-trigger LH noted that an LH ≤ 15 IU/L resulted in a lower oocyte yield than cycles with a serum LH above 15 IU/L,

but no differences in oocyte maturity [72]. Predicting the probability of not obtaining oocytes after GnRHa is therefore very important to decide whether to proceed with retrieval or administer a rescue hCG dose. Meyer et al. examined risk factors for a low post-trigger LH ≤ 15 IU/L and found that patients with a suboptimal response were more likely to have low serum FSH (<0.1 mIU/mL) and LH levels (<0.1 mIU/mL) at the start of the cycle, low LH on the day of trigger (<0.5 mIU/mL), and were more likely to be on long-term oral contraception [33]. These results are further supported by a systematic review by Herman et al. [73]. Low serum LH at the start of stimulation as a risk factor for suboptimal oocyte yield is further supported by a retrospective cohort study by Popovic-Todorovic et al., which demonstrated that patients with an LH <0.1 IU/L had a 45.2% risk of suboptimal response [74]. Low baseline serum FSH and LH may represent women on long-term oral contraceptive pills or that have some form of dysfunction in the hypothalamic–pituitary axis. Other studies have also confirmed that low body mass index and low baseline LH are risk factors for an inadequate response to GnRHa trigger [75].

There are published reports of failed oocyte maturation after GnRHa trigger, often detected with a low serum LH on the day after trigger [76]. Rates of EFS after GnRHa were between 1.4% and 3.5% in two studies, which did not differ significantly from rates of EFS after hCG trigger (0.1%–2%) [54, 77–82]. It has been estimated that <2%–2.7% [75, 83] of patients triggered with GnRHa have an inadequate response, defined as LH <15 IU/L, and require retrigger with hCG. A survey of practitioners from clinics worldwide reported that 11% of physicians who use a GnRHa trigger have encountered a case of EFS [84]. In lieu of cancelling the cycle or immediate retriggering with hCG, the patient can proceed with unilateral follicle aspiration and if there are no oocytes, retrigger with hCG and repeat oocyte retrieval of the contralateral ovary 34 hours later [76].

Post-trigger serum levels of LH and progesterone drawn approximately 12 hours after trigger can provide warning for a failed endogenous response to the trigger injection, and

TABLE 44.3 Trials Demonstrating Effect of GnRHa Trigger on Oocyte Yield and Maturation

Study	Oocyte Yield		Oocyte Maturation (%)	
	GnRHa	hCG	GnRHa	hCG
Fauser et al. 2002	8.7 \pm 4.5	8.3 \pm 3.3	87 \pm 17	86 \pm 17
Humaidan et al. 2005	8.4	9.7	84 \pm 18	68.0 \pm 22.0*
Kolibianakis et al. 2005	10.2 \pm 7.0	10.6 \pm 6.3	73.5 \pm 4.5	78.7 \pm 3.3
Babayoff et al. 2006	19.8 \pm 2.5	19.5 \pm 1.9	89.4	84.1
Acevedo et al. 2006	9.1 \pm 4.0	10.3 \pm 6.3	70	76
Engmann et al. 2008	20.2 \pm 9.9	18.8 \pm 10.4	81.0 \pm 16.3	83.8 \pm 13.2
Galindo et al. 2009	11.4 \pm 6.4	12.0 \pm 6.3	67.1 \pm 20.9	67.2 \pm 20.4
Melo et al. 2009	17.1 \pm 2.7	18.7 \pm 3.1	75.4	78.6
Sismanoglu et al. 2009	38.2 \pm 14.5	36.6 \pm 11.1	81.1	79.4
Papanikolaou et al. 2011	11.7 \pm 1.9	13.8 \pm 1.8	67.5	60.1
Sahin et al. 2015	12.8 \pm 0.6	7.6 \pm 0.2*	75.8	88.2*
Christopoulos et al. 2016	18	15*	83.3	86.7*
Maslow et al. 2020	18.3 \pm 12.1	12.5 \pm 7.7*	72.6	67.2*
Deepika et al. 2021	23.5 \pm 7.8	20.8 \pm 5.4*	81.3	67.8*
Yilmaz et al. 2021	15	14	92	78.6

* Findings statistically significantly different.

intervention may be possible. If there is no LH surge and/or progesterone rise after GnRHa trigger, repeat trigger with hCG and oocyte retrieval 35 hours later have been shown to result in successful retrieval of oocytes [54]. If there is a suboptimal LH rise with values less than 15 IU/L, repeat trigger with hCG can be given as soon as possible to proceed with retrieval as planned or the cycle may be cancelled.

Addition of standard or low-dose hCG to GnRHa trigger in a “dual trigger” protocol may improve the number and proportion of mature oocytes [66, 85–88] and high-quality embryos [87–91], and has been adopted for wide use in some clinics to reduce the chances of EFS after either hCG or GnRHa only trigger [33, 92]. A randomized controlled trial comparing hCG 10,000 IU with GnRHa plus hCG 10,000 IU demonstrated higher numbers of oocytes and mature oocytes as well as clinical pregnancy and live birth rates in the dual trigger group compared to hCG alone [93]. These findings were further supported in a systematic review and meta-analysis of randomized trials investigating the effects of dual trigger with standard dose hCG and GnRHa and found a significantly higher live birth rate in the dual trigger group [92].

Other approaches have been investigated to improve oocyte maturity in women with previous high proportion of immature oocytes and reduce the risk of EFS [93, 94]. A study by Zilberman et al. investigated the effects of dual trigger with GnRHa administered 40 hours prior to oocyte retrieval combined with hCG administered 34 hours prior to oocyte retrieval in women with previous high proportion of immature oocytes [94]. They found a significantly higher number of MII oocytes, a higher proportion of MII oocytes/oocyte retrieved, and a higher number of high-quality embryos in the group receiving dual GnRHa and hCG trigger compared to hCG trigger alone [94]. However, the use of adjuvant hCG in addition to GnRHa trigger should be used with caution in patients at high risk of OHSS development [52, 95].

Luteal phase steroid profile after natural cycle and GnRHa trigger

In the luteal phase of a natural menstrual cycle, LH acts as a luteotropic hormone which supports the growth and function of the corpus luteum and steroidogenesis after ovulation [96]. Luteal phase LH increases growth factors and cytokines necessary for implantation, such as VEGF and fibroblast growth factor 2 [97, 98]. The circulating LH also promotes action via LH receptors located outside the ovary: in the endometrium, fallopian tubes, early fetal cells, placenta, and numerous other tissues. As a result, LH has many regulatory roles before and during pregnancy, including the synthesis of prostaglandins and tubal glycoproteins, stimulation of embryonic growth in the tube, and initiation and maintenance of pregnancy in the uterus [99].

In a natural cycle, if pregnancy does not occur and hCG is not available to continue to support the function of the corpus luteum, withdrawal of LH will result in luteolysis and then menses. In the setting of IVF, use of any trigger for oocyte maturation without luteal phase support in an IVF cycle using a GnRH antagonist will significantly reduce the length of the luteal phase [16]. The median duration of the luteal phase after GnRHa trigger may be as short as nine days compared to 13 days after hCG trigger [16]. LH is secreted in a pulsatile manner and although the number of LH pulses is similar on the day of oocyte retrieval and 48 hours later, there was a trend towards decreasing amplitude, and the basal

LH secretory rate was significantly lower 48 hours after oocyte retrieval compared with the day of oocyte retrieval when GnRHa was used (0.39 ± 0.036 IU/L/min vs 0.0042 ± 0.0027 IU/L/min) [100]. This rapid decrease in LH secretion with GnRHa explains the shorter duration of the luteal phase after GnRHa trigger compared to hCG trigger. The duration of the LH surge after GnRHa trigger is short with a median serum LH <2 IU/L on day 4 after trigger, and a shortened surge correlates with decreased production of progesterone throughout the luteal phase [3, 16]. Serum levels of progesterone and oestrogen throughout the luteal phase are significantly lower with GnRHa trigger than after an hCG trigger [3, 14, 16, 101].

The shortened duration of the LH surge after GnRHa trigger is enough to induce maturation of oocytes, but not sufficient to induce and maintain adequate corpora lutea to resemble a natural luteal phase [40, 102, 103]. After the trigger, GnRHa may partially downregulate the pituitary, continuing to inhibit the release of endogenous LH [104]. By an additional mechanism common to most IVF protocols, supra-physiologic levels of progesterone and oestrogen from ovarian stimulation also suppress LH release from the pituitary [16, 105]. All these factors together result in early luteolysis. Unfortunately, even if pregnancy does occur after GnRHa trigger, the luteolytic process is profound and significant enough that the corpora lutea cannot reliably be rescued by the time endogenous hCG from an implanting embryo is detected in the circulation [106]. Nevo et al. measured levels of inhibin A and pro- α C, which are markers of corpus luteum function, and found that in the late luteal phase, the onset of pregnancy and the presence of hCG did not correlate with an increase in these corpora lutea markers [106]. In fact, endometrial gene expression studies have shown significant alteration in gene expression after GnRHa trigger [107, 108]. Furthermore, Kaye et al. demonstrated prorenin and 17α -hydroxyprogesterone, which indicate corpus luteum function, peak at five days and two days, respectively, and nadir at nine days after GnRHa trigger. This finding demonstrates that corpus luteum function continues, at least initially, despite administration of GnRHa [101].

The preceding holds true in the normogonadotrophic woman, but it should also be noted that the luteal phase of select patients may differ somewhat in a way that alters the hormonal milieu after GnRHa trigger. It is possible that PCOS patients may have an elevated serum LH through both the follicular and luteal phases compared with normogonadotrophic women; additionally, they may have decreased sensitivity at the hypothalamus to inhibition by ovarian steroids such as progesterone [109]. These factors may contribute to a favourable response after GnRHa trigger and should be considered when discussing the luteal phase steroid profile of the infertile and sub-fertile population.

Strategies for modifying the luteal phase and pregnancy rates

After early studies suggested that the luteal phase was suboptimal to achieve excellent clinical and ongoing pregnancy rates after GnRHa trigger [110], numerous strategies have been proposed to modify the standard luteal support in order to increase pregnancy rates after fresh embryo transfer without significantly increasing the risk for OHSS. These modifications include intensive exogenous luteal phase steroid support and close monitoring of serum oestrogen and progesterone levels [19, 56, 111, 112], an adjuvant low dose of hCG given at the time of GnRHa trigger or at

the time of retrieval [21, 22, 24, 112–116], or luteal phase recombinant LH administration [28].

Standard luteal support

As mentioned earlier, supra-physiologic levels of steroid hormones during a stimulated cycle provide negative feedback on the pituitary, resulting in a decrease in endogenous LH and the potential for early luteolysis [105]. As a result, standard luteal support is generally given during IVF cycles. Standard luteal-phase support used after GnRHa may vary between centres but may include a regimen of progesterone alone or in combination with oestrogen supplementation.

In the mid-2000s a meta-analysis reviewed the outcomes after GnRHa trigger with the use of conventional luteal support. The review included three publications; two were stopped early due to significant differences in clinical outcomes in favour of the hCG groups [14, 15, 20]. Their luteal support protocols differed but primarily involved vaginal micronized progesterone with or without oral oestrogen starting after transfer and discontinued between the first positive pregnancy test and seven weeks of gestation. The meta-analysis revealed a clinical pregnancy rate of 7.9% in the GnRHa group compared to 30.14% in the hCG group [110]. Early pregnancy loss rates were also noted to be higher than those of the hCG group [110].

Intensive luteal support

Knowing that the serum levels of oestrogen and progesterone after GnRHa trigger decrease significantly, a strategy to improve the dysfunctional luteal phase includes a more intensive luteal phase support protocol. This has been described as supplementation with both oestrogen and progesterone in addition to close monitoring of serum steroid levels to adjust doses as necessary. The supplementation protocol that has been described by Engmann et al. [19] in a randomized controlled study of 66 PCOS or high responding patients begins with initiation on the day after retrieval of 50 mg IM progesterone daily and three 0.1 mg oestradiol transdermal patches placed every other day (Figure 44.2). Serum levels of oestradiol and progesterone were evaluated on three and seven days after oocyte retrieval and weekly thereafter, with continuation of IM progesterone and transdermal oestrogen supplementation until approximately 10 weeks gestational age. Based on serum levels, doses of IM progesterone were increased to a maximum of 75 mg daily, with the addition of micronized vaginal progesterone daily as needed to maintain serum

progesterone above 20 ng/mL. Similarly, oestrogen patches could be increased to four 0.1 mg patches every other day, with addition of oral micronized oestradiol (2 mg to 8 mg) daily to maintain serum oestradiol above 200 pg/mL [19]. This study, which compared an intensive luteal phase support after GnRHa trigger with standard luteal phase support after an hCG trigger, resulted in a 53% ongoing pregnancy rate comparable to 48.3% in the hCG group.

These results have been corroborated by other investigators [56, 95, 114]. Imbar et al. [56] described an intensive luteal support of 50 mg IM progesterone in oil as well as 6 mg oral oestradiol started on the day of retrieval and continued until 10 weeks of gestation; with 70 patients in the study arm, a clinical pregnancy rate of 37% and live birth rate of 27.1% was found, comparable to patients who underwent cryopreservation with subsequent freeze-thaw embryo transfer. In a retrospective cohort study, Iliodromiti et al. noted equivalent live birth rates of 29% in both GnRHa and hCG groups [95]. Shapiro et al. reported a 50% ongoing pregnancy rate in GnRHa trigger patients receiving enhanced luteal support, significantly improved over women with agonist trigger alone and standard luteal support (25.3% ongoing pregnancy rate) and comparable to a 57.7% ongoing pregnancy rate in dual trigger patients, described next [114].

The availability of IM progesterone is not universal and must be considered when planning to provide intensive luteal supplementation. In protocols utilizing an hCG trigger, studies suggest that there is no superiority of IM progesterone over vaginal progesterone [117, 118]; however, this may be essential after GnRHa trigger.

Adjuvant low-dose hCG

As it is the activity of LH in the luteal phase that supports steroidogenesis from the corpus luteum, a number of strategies have been described to restore or replace the function of LH in the luteal phase after use of a GnRHa trigger, often in addition to providing the luteal phase steroids exogenously. The use of any dose of hCG in addition to GnRHa trigger should be used cautiously since it may potentially increase the risk of OHSS development.

Dual trigger with hCG

The concept of dual trigger with low-dose hCG and GnRHa is to provide a small amount of hCG to help rescue the corpora lutea by providing the additional signal necessary for adequate luteinization. Shapiro et al. described a dual trigger protocol with an hCG

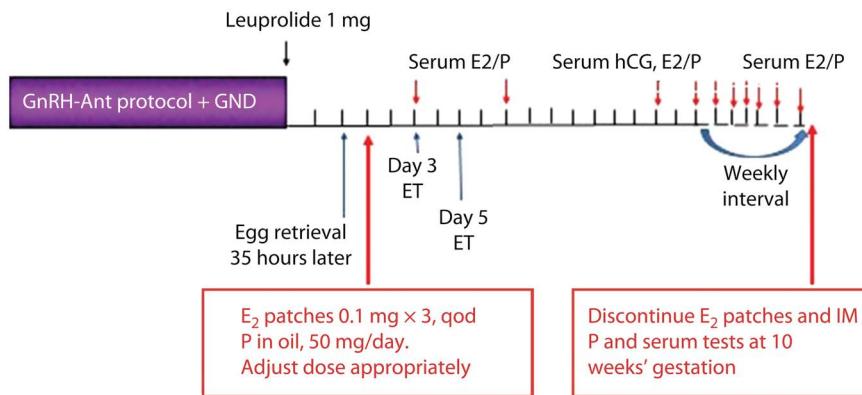


FIGURE 44.2 Components of intensive luteal phase support. E2 = estradiol, P = progesterone, IM = intramuscular, qod = every other day

dose ≤ 33 IU/kg body weight (ranging between 1000 and 2500 IU) with an ongoing pregnancy rate of 53.3% [113]. The same group later published another similar study reporting a 57.7% ongoing pregnancy rate with one case of clinically significant OHSS [114]. In order to simplify the regimen and reduce the risk of OHSS, Griffin et al. recommended low hCG dose of 1000 IU given with GnRHa trigger and intensive luteal steroid support. The live birth rate of 52.9% was significantly higher than the 30.9% rate noted after GnRHa trigger alone in patients with serum E2 < 4000 pg/mL [21]. The authors noted one case of mild OHSS in the dual trigger group versus no OHSS in the GnRHa alone group [21]. The added benefit for the dual trigger is to serve as a “back-up” in the case of GnRHa trigger failure, in addition to the potential for more mature oocytes and higher quality embryos as discussed earlier [33].

Adjuvant hCG at time of oocyte retrieval

Humaidan et al. have described in multiple studies the use of a single bolus of 1500 IU hCG given on the day of oocyte retrieval, typically within one hour of retrieval, in addition to standard luteal-phase support [22–24]. It has been previously shown that the granulosa/luteal cells are viable and able to respond to hCG on the day of retrieval [49]. A randomized trial of 302 IVF cycles comparing one bolus of hCG 1500 IU after GnRHa trigger with an hCG trigger showed no significant difference in delivery rates of 24% versus 31%, respectively [24]. These results were corroborated by a retrospective cohort study by Karacan et al. which demonstrated similar live birth rates (45.9% vs 43.8%) among patients who underwent a fresh embryo transfer after receiving GnRHa trigger and 1500 IU hCG bolus at the time of oocyte retrieval compared to patients who received GnRHa trigger and cryopreserved all embryos with subsequent thawed embryo transfer [119]. The use of a 1500 IU hCG bolus at the time of oocyte retrieval shows promise despite a possible increased risk of OHSS.

Large retrospective studies report clinical pregnancy rates of 41.8%–52.1% while maintaining low rates of severe OHSS [52, 112]. A prospective randomized double-blind trial of hCG at the time of GnRHa trigger versus hCG at the time of oocyte retrieval showed no significant difference in live birth rates between the groups, but a slightly higher, although not significant, incidence of OHSS among patients who received hCG at the time of oocyte retrieval (9.7% vs 3.8%).¹¹⁶ These results are corroborated by a randomized controlled trial that found similar ongoing pregnancy rates (49% vs 50%) among patients who received GnRHa trigger plus 1500 IU hCG at the time of oocyte retrieval and those who received hCG trigger in the setting of low rates of OHSS [120]. Despite the low occurrence of OHSS in multiple studies utilizing hCG at the time of oocyte retrieval, there have been reports of severe OHSS in this setting. Radesic et al. reported one case of severe OHSS among 71 women at high risk of OHSS receiving 1500 IU hCG within one hour after vaginal oocyte retrieval [52]. Iliodromiti et al. reported two cases of severe OHSS out of 275 cycles using the same trigger protocol [95]. However, Seyhan et al. evaluated 23 women at high risk of OHSS with mean E2 4891 pg/mL on day of trigger who received a GnRHa trigger and hCG 1500 IU administered within one hour of oocyte retrieval and reported a high severe OHSS rate of 26% (6/23) [121].

Adjuvant hCG two days following oocyte retrieval

A proof-of-concept study administered a 1500 IU hCG bolus two days after oocyte retrieval without additional progesterone supplementation. Mean progesterone levels 14 days after oocyte

retrieval were similar to the control group that received hCG trigger and conventional progesterone luteal support. Ongoing pregnancy, miscarriage, and live birth rates were comparable between the two groups and there were no cases of OHSS [122]. These findings were corroborated by Kol et al. who found similar pregnancy rates and higher oestradiol levels two weeks after oocyte retrieval in the group receiving a 1500 IU hCG bolus two days following oocyte retrieval compared with the hCG trigger group [123]. This suggests administration of hCG two days following oocyte retrieval may rescue the corpora lutea and allow for ongoing steroidogenesis at sufficient levels to support an early pregnancy. However, more studies are required to further investigate this protocol.

Very low hCG dose

A very low dose of daily hCG has also been described which resulted in good clinical pregnancy rates by rescuing corpora lutea function without the need for additional supplementation of progesterone or oestradiol. Recombinant hCG 125 IU was given daily starting on either day 2 or day 6 of stimulation and continued daily throughout the luteal phase [124, 125]. This protocol, in a proof-of-concept study of normal responders, showed significantly higher luteal progesterone levels without exogenous supplementation compared with a standard luteal phase protocol, and pregnancy outcomes were the same in the study arm versus the control arm using standard luteal support [125]. Additional confirmatory studies are necessary before incorporating this approach into common practice. Very low doses of hCG are not currently commercially available in most countries.

Recombinant LH

When recombinant LH is available, this can also be considered for luteal phase supplementation, perhaps with the benefit of a shorter half-life than hCG to further minimize OHSS risk. However, only one study has been published describing the dose and timing of its use in normal responder patients. Although comparable delivery rates were noted and there were no cases of OHSS compared to an hCG trigger control group, these findings have not been corroborated [28].

Luteal GnRHa

Another concept for luteal support was investigated by Fusi et al. and utilized triptorelin 0.1 mg every other day, alternating with progesterone in oil, beginning on the day of embryo transfer. It was theorized that repeated doses of triptorelin would stimulate recurrent LH surges to support the corpus luteum, however serum LH was not monitored after the administration of luteal doses of triptorelin. In this study, there was no significant difference in implantation, clinical pregnancy, and ongoing pregnancy rates in the GnRHa trigger group with luteal GnRHa support compared with the hCG trigger group. Moreover, there was a statistically significant lower rate of OHSS in the triptorelin trigger group compared to the hCG trigger group [126]. Further research is needed to investigate the potential use of luteal GnRHa.

Luteal coasting

Using a similar strategy to coasting at the end of stimulation in high responder patients, Kol et al. obtained pregnancies after fresh transfer through luteal coasting after trigger [127]. In their case series of 21 high-responder patients, no luteal phase steroid supplementation was provided unless monitored serum progesterone levels dropped significantly, at which time a bolus of 1500 IU hCG was administered [127]. This approach individualizes the

luteal supplementation, providing exogenous support when indicated and avoiding excessive stimulus when the risk for OHSS is elevated. A similar strategy for individualized luteal support was advocated in a case series by Lawrenz et al. who reported luteal levels of progesterone ranging from 14 to 43.69 ng/mL despite all patients having 20 oocytes retrieved after GnRHa trigger, which demonstrates that luteolysis after GnRHa trigger varies between individuals and argues for individualized luteal support [128]. In a small case series by the same group, it was demonstrated that ongoing pregnancies can be achieved when early luteal progesterone levels decrease to <15 ng/mL as long as a 1500 IU hCG bolus is administered, with an ongoing pregnancy rate of 66.7% (2/3 patients) [129]. However, it is important to be cautious when interpreting a single serum level, as a previous study showed that endogenous progesterone levels can vary by eightfold within 90 minutes in the same study subject [130]. This strategy of individualized luteal support depending on progesterone levels requires additional studies to confirm its efficacy.

Cycle segmentation: Cryopreservation of all oocytes or embryos

In an attempt to overcome the suboptimal luteal phase after GnRHa trigger, a freeze-all policy with transfer after thaw during a subsequent cycle has been proposed [131–135]. Not only can segmentation of the IVF process avoid the early or late-onset OHSS in high responders, but also implantation and pregnancy rates can be optimized. Manzanares et al. reported a 33% pregnancy rate in PCOS patients with previous cycle cancellations after freezing all embryos with a subsequent thaw and transfer cycle [135]. However, the study did not include a control group. Garcia-Velasco reported a 50% clinical pregnancy rate for patients at high risk for OHSS who opted to freeze all oocytes and undergo thaw and transfer of embryos in a subsequent natural cycle, compared to 29.5% in high risk patients after coacting

and fresh embryo transfer [132]. The segmentation approach has become a feasible option in view of studies that have shown excellent pregnancy rates after freeze-all cycles. Furthermore, a retrospective cohort study by Makhijani et al. showed similar implantation, clinical pregnancy, clinical loss, and live birth rates between patients who received GnRHa and hCG triggers and underwent subsequent thaw and transfer cycles of euploid embryos [136]. Overall, the cycle segmentation approach shows positive results; however, factors associated with the cost of additional frozen embryo transfer cycles must be considered and may be best suited for specific clinical situations [137].

Individualization of protocols to improve conception rates

In view of the different approaches that have been recommended by various researchers, it is important to develop an individualized approach to managing the luteal phase and optimizing conception rates without increasing the risk of OHSS development (Figure 44.3). Previous studies have attempted to determine the predictors of clinical outcomes in an attempt to formulate management guidelines that are tailored to a patient's response. One study found the most important predictors of pregnancy success after GnRHa trigger and intensive luteal support were a peak E2 ≥ 4000 pg/mL and an elevated LH on the day of trigger [138], suggesting that the elevated LH at trigger functions to rescue some corpora lutea and results in increased rates of conception. In that study, women with peak serum E2 of ≥ 4000 pg/mL had a significantly higher clinical pregnancy rate of 53.6%, compared with 38.1% in women with peak E2 of <4000 pg/mL. A study by Griffin et al. showed that the use of a dual trigger GnRHa with low dose hCG of 1000 IU results in a significantly higher live birth rate compared with GnRHa trigger alone in women with peak E2 <4000 pg/mL [21]. Additionally, a prospective randomized double-blind clinical trial by Engmann et al. demonstrated

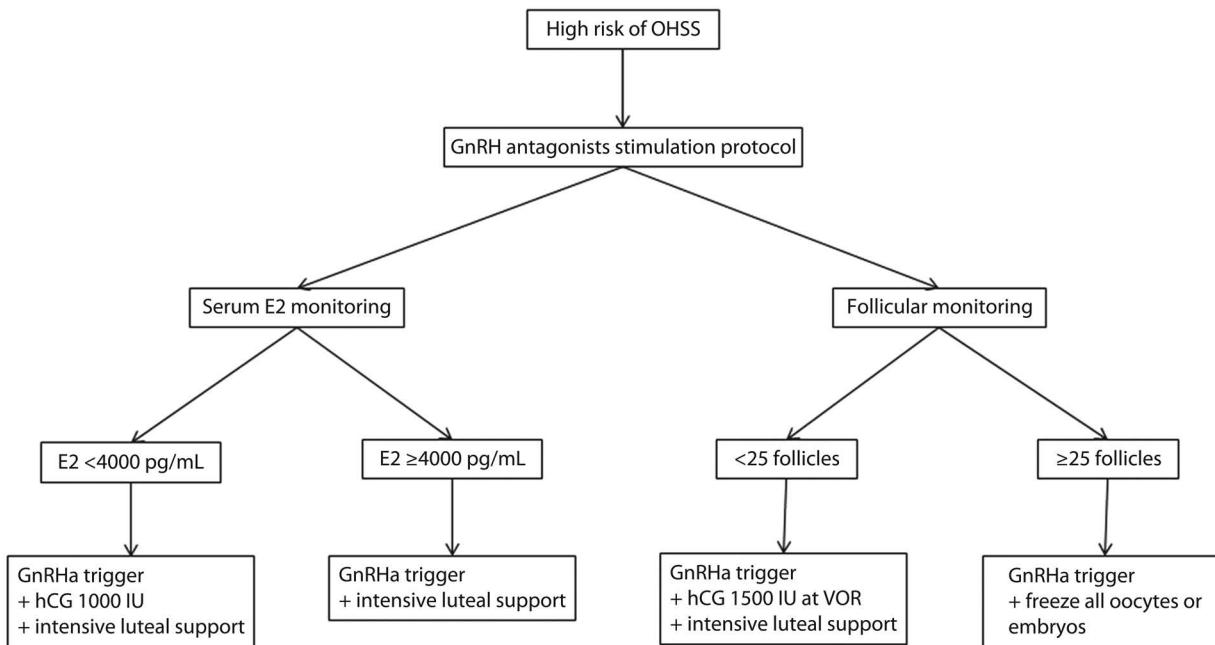


FIGURE 44.3 Suggested luteal-phase support protocols by high-responder characteristics. VOR = vaginal oocyte retrieval

similar live birth rates between patients who received GnRHa trigger and 1000 IU hCG at the time of trigger versus 1500 IU hCG at the time of oocyte retrieval.¹¹⁶

For patients with a peak serum E2 \geq 4000 pg/mL, intensive luteal phase supplementation with progesterone and oestradiol may be all that is necessary to optimize conception rates. However, for women with a peak E2 less than this threshold [21], an adjuvant low dose of hCG may have an additional benefit on pregnancy rates.

The alternative criteria are the number of follicles on the day of trigger and/or the number of small to intermediate size follicles, to determine whether to use an hCG bolus of 1500 IU on the day of retrieval or to freeze all oocytes/embryos [139]. Seyhan et al. proposed that women with more than 18 follicles measuring between 10 and 14 mm should avoid hCG bolus and undergo oocyte/embryo cryopreservation based on a risk of severe OHSS of 26% after the use of 1500 IU bolus at the time of retrieval [121]. Other studies have also suggested that women with more than 25 follicles greater than 11 mm in diameter should be considered for a freeze-all strategy in order to completely eliminate the risk of OHSS [115, 140].

Ovarian hyperstimulation syndrome

The short duration of the LH surge results in inadequate corpus luteum formation and early corpus luteum demise after GnRHa trigger, which has been shown to be effective in the prevention of OHSS. Table 44.4 [141] lists various publications regarding OHSS rates after GnRHa trigger compared to hCG trigger. Overall, the elimination of OHSS is noted after GnRHa trigger, corroborated by a recent Cochrane review from Youssef et al. [141, 142].

Despite the use of a GnRHa for trigger, there are still a few cases of moderate to severe OHSS that persist. Some of these

cases result from the use of low-dose hCG supplementation in the luteal phase. However, some cases of OHSS after the use of GnRHa alone have been reported and require additional exploration of compound aetiologies, including mutations in the GnRH, FSH, or LH receptors, or variations in the genes for VEGF, its receptor, or other important vasoactive substances. Ling et al. described a case of early onset severe OHSS occurring shortly after oocyte retrieval in a woman with an AMH of 64.5 ng/mL who received a leuprolide trigger as well as a freeze-all segmentation strategy [50]. Fatemi et al. also described two cases of severe OHSS after GnRHa trigger alone without any adjuvant hCG and who did not have fresh embryo transfers [143]. Activating mutations of the FSH receptor or the GnRH receptor could predispose patients to OHSS despite the use of a GnRHa trigger [143]. Moreover, the addition of any dose of adjuvant hCG to GnRHa trigger may be associated with a significantly higher risk of OHSS compared with GnRHa trigger alone [121, 144].

Use of GnRHa trigger in specific clinical situations

Freeze-all for PGT cycles

GnRHa trigger may be especially applicable in freeze-all cycles in which PGT with a subsequent thaw and transfer cycle is planned because luteolysis in the current cycle is not of concern. A retrospective cohort study by Thorne et al. demonstrated similar euploidy rates per embryo biopsied and per oocyte retrieved in the GnRHa and hCG trigger groups [145]. In a follow-up study by the same group, there was no difference in the ongoing pregnancy/live birth rates when single euploid blastocysts were thawed and transferred to patients who had received GnRHa versus hCG trigger (64.1% vs 65.3%, P = 0.90) [136]. Together, these studies indicate that GnRHa trigger does not adversely affect oocyte/embryo

TABLE 44.4 OHSS Incidence after GnRHa Triggering of Final Oocyte Maturation versus hCG Triggering in Published Trials

Studies	Study Design	OHSS Risk	Agonist Triggering Arm	hCG Triggering Arm
Fresh IVF Cycles with Embryo Transfer (ET)				
Fauser et al. (2002) [20]	RCT	Normal	0% (0/32)	0% (0/15)
Humaidan et al. (2005) [14]	RCT	Normal	0% (0/55)	0% (0/67)
Kolibianakis et al. (2005) [15]	RCT	Normal	0% (0/52)	0% (0/54)
Pirard et al. (2006) [154]	RCT	Normal	0% (0/6)	0% (0/6)
Humaidan et al. (2006) [23]	RCT	Normal	0% (0/13)	0% (0/15)
Babayof et al. (2006) [55]	RCT	High	0% (0/15)	31.0% (4/13)
Engmann et al. (2008) [19]	RCT	High	0% (0/33)	31.0% (10/32)
Humaidan et al. (2010) [24]	RCT	Normal/high	0% (0/152)	2.0% (3/150)
Papanikolaou et al. (2011) [28]	RCT	Normal	0% (0/17)	0% (0/18)
Christopoulos et al. (2016) [70]	Retrospective cohort	High	0.3% (1/382)	13.4% (26/194)
Donor IVF Cycles (No ET)				
Acevedo et al. (2006) [67]	RCT	Normal	0% (0/30)	17.0% (5/30)
Galindo et al. (2009) [11]	RCT	Normal	0% (0/106)	8.5% (9/106)
Melo et al. (2009) [27]	RCT	Very high	0% (0/50)	4.0% (2/50)
Sismanoglu et al. (2009) [68]	RCT	Very high	0% (0/44)	6.8% (3/44)
Total Embryo Freezing (No ET)				
Griesinger et al. (2007) [155]	Observational	Very high	0% (0/20)	-
Manzanares et al. (2010) [135]	Observational	Very high	0% (0/42)	-

Source: From [141], with Permission, with updated material.

quality and leads to comparable live birth rates as cycles triggered with hCG.

Oocyte donation cycles

Several retrospective cohort and prospective randomized trials in donor oocyte cycles have shown no differences in the number of oocytes retrieved, proportion of mature oocytes, fertilization rates, implantation rates, pregnancy rates, and live birth rates between cycles resulting from GnRHa compared to hCG triggers [25, 60, 63, 64, 138, 142, 146]. In recipient patients, pregnancy rates ranged from 38%–55% (compared to 38%–59% after hCG trigger) with a miscarriage rate of 15.4%–22.2% [27, 67, 147].

Use of the GnRHa trigger in oocyte donors with normal or high responses to ovarian stimulation has a clear advantage in the prevention of OHSS [17, 27, 148]. Randomized clinical trials [27, 67, 142, 147] as well as retrospective cohort studies [63, 64, 146] comparing GnRHa and hCG trigger have shown a significant reduction in the risk of OHSS. Rates of OHSS in the hCG trigger arms ranged from 4.0%–17.0% in a population of women undergoing elective controlled ovarian stimulation for the purpose of oocyte donation. The GnRHa trigger arms had no cases of moderate or severe OHSS out of 186 women reviewed by Youssef et al. [142].

Breast cancer patients

Patients diagnosed with oestrogen receptor-positive breast cancer may elect to undergo cryopreservation of embryos or oocytes and may be good candidates for GnRHa trigger. A study by Oktay et al. found that, after stimulation with gonadotropins and an aromatase inhibitor to minimize systemic oestrogen exposure, GnRHa trigger not only minimized the risk for OHSS such that patients can recover quickly after stimulation to proceed with cancer therapy, but GnRHa trigger resulted in significantly lower serum oestradiol levels in the luteal phase [31].

Safety of GnRHa use

When compared with an hCG trigger, maternal and neonatal outcomes are likely equivalent, but there is little published evidence. In a retrospective study, Budinetz et al. found no significant differences in the rate of congenital anomalies between GnRHa and hCG trigger (6.6% vs 9.2%) [149]. There were also no differences in the maternal complications (27.6% vs 20.8%) or minor or major neonatal complications (19.7% vs 20.0%) between the GnRHa and hCG trigger groups [149].

Other advantages

Multiple studies have reported improvements in patient comfort after GnRHa trigger compared to hCG [19, 64, 150]. GnRHa trigger alters the uncomfortable characteristics common in the luteal phase, including smaller ovarian volumes and decreased fluid in the pelvis, thus reducing abdominal bloating and pain. The duration of the uncomfortable luteal phase is also shortened with earlier menses, which can affect patient satisfaction, especially for oocyte donors and women who are not planning a fresh transfer [19, 64, 150]. Concomitant with the reduction in ovarian size is a decreased risk of ovarian torsion in patients receiving GnRHa compared with hCG trigger [151].

GnRHa trigger in addition to a standard dose of hCG has the advantage of providing an additional option for patients with a history of immature oocytes, empty follicle syndromes, or low

fertilization after hCG trigger. The dual surge of LH and FSH may have benefits in its resemblance to a natural cycle surge that could assist in strategies to prevent recurrent failed cycles [85, 152, 153].

Conclusion

The increasingly successful use of the GnRHa trigger has changed the practice and goals of assisted reproductive technology. Since the Copenhagen GnRH Agonist Triggering Workshop Group meeting in 2009, which noted the remarkable prevention of OHSS after use of a GnRHa trigger when appropriate, there has been tremendous research in optimizing luteal support [141]. A new definition of success in assisted reproductive technology should be the achievement of pregnancy, without OHSS, that results in a healthy singleton live birth at term. Additionally, reporting systems can be modified to incorporate OHSS in success rates which could encourage practices to take additional steps to avoid OHSS, particularly among high responders such as women with PCOS, women undergoing elective cryopreservation, and oocyte donors for whom safety is the primary concern.

References

- Emperaire JC. [Therapeutic induction of ovulation: Towards the replacement of hCG with LH]. *Contracept Fertil Sex*. 1994;22(7–8):459–67.
- Gonen Y, Balakier H, Powell W, et al. Use of gonadotropin-releasing hormone agonist to trigger follicular maturation for in vitro fertilization. *J Clinic Endocrinol Metabol*. 1990;71(4):918–22.
- Itskovitz J, Boldes R, Levron J, et al. Induction of preovulatory luteinizing hormone surge and prevention of ovarian hyperstimulation syndrome by gonadotropin-releasing hormone agonist. *Fertil Steril*. 1991;56(2):213–20.
- Itskovitz-Eldor J, Levron J, Kol S. Use of gonadotropin-releasing hormone agonist to cause ovulation and prevent the ovarian hyperstimulation syndrome. *Clinic Obstetr Gynecol*. 1993;36(3):701–10.
- Lanzone A, Fulghesu AM, Apa R, et al. LH surge induction by GnRH agonist at the time of ovulation. *Gynecol Endocrinol*. 1989;3(3):213–20.
- Lewit N, Kol S, Manor D, et al. Comparison of gonadotrophin-releasing hormone analogues and human chorionic gonadotrophin for the induction of ovulation and prevention of ovarian hyperstimulation syndrome: A case-control study. *Hum Reprod*. 1996;11(7):1399–402.
- Segal S, Casper RF. Gonadotropin-releasing hormone agonist versus human chorionic gonadotropin for triggering follicular maturation in in vitro fertilization. *Fertil Steril*. 1992;57(6):1254–8.
- van der Meer S, Gerris J, Joostens M, et al. Triggering of ovulation using a gonadotrophin-releasing hormone agonist does not prevent ovarian hyperstimulation syndrome. *Hum Reprod*. 1993;8(10):1628–31.
- Albano C, Smitz J, Camus M, et al. Comparison of different doses of gonadotropin-releasing hormone antagonist cetrorelix during controlled ovarian hyperstimulation. *Fertil Steril*. 1997;67(5):917–22.
- Mannaerts B, Abyholm T, De Jong D, et al. A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist ganirelix (Org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon). The ganirelix dose-finding study group. *Hum Reprod*. 1998;13(11):3023–31.
- Itskovitz-Eldor J, Kol S, Mannaerts B. Use of a single bolus of GnRH agonist triptorelin to trigger ovulation after GnRH antagonist ganirelix treatment in women undergoing ovarian stimulation

- for assisted reproduction, with special reference to the prevention of ovarian hyperstimulation syndrome: Preliminary report: Short communication. *Hum Reprod.* 2000;15(9):1965–8.
12. Reissmann T, Felberbaum R, Diedrich K, et al. Development and applications of luteinizing hormone-releasing hormone Antagonists in the treatment of infertility: An overview. *Hum Reprod.* 1995;10(8):1974–81.
 13. Porter RN, Smith W, Craft IL, et al. Induction of ovulation for in-vitro fertilisation using buserelin and gonadotropins. *Lancet.* 1984;2(8414):1284–5.
 14. Humaidan P, Bredkjaer HE, Bungum L, et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomized study. *Hum Reprod.* 2005;20(5):1213–20.
 15. Kolibianakis EM, Schultze-Mosgau A, Schroer A, et al. A lower ongoing pregnancy rate can be expected when GnRH agonist is used for triggering final oocyte maturation instead of HCG in patients undergoing IVF with GnRH antagonists. *Hum Reprod.* 2005;20(10):2887–92.
 16. Beckers NG, Macklon NS, Eijkemans MJ, et al. Nonsupplemented luteal phase characteristics after the administration of recombinant human chorionic gonadotropin, recombinant luteinizing hormone, or gonadotropin-releasing hormone (GnRH) agonist to induce final oocyte maturation in in vitro fertilization patients after ovarian stimulation with recombinant follicle-stimulating hormone and GnRH antagonist cotreatment. *J Clinic Endocrinol Metabol.* 2003;88(9):4186–92.
 17. Bodri D, Sunkara SK, Coomarasamy A. Gonadotropin-releasing hormone agonists versus antagonists for controlled ovarian hyperstimulation in oocyte donors: A systematic review and meta-analysis. *Fertil Steril.* 2011;95(1):164–9.
 18. DiLuigi AJ, Engmann L, Schmidt DW, et al. Gonadotropin-releasing hormone agonist to induce final oocyte maturation prevents the development of ovarian hyperstimulation syndrome in high-risk patients and leads to improved clinical outcomes compared with coasting. *Fertil Steril.* 2010;94(3):1111–4.
 19. Engmann L, DiLuigi A, Schmidt D, et al. The use of gonadotropin-releasing hormone (GnRH) agonist to induce oocyte maturation after cotreatment with GnRH antagonist in high-risk patients undergoing in vitro fertilization prevents the risk of ovarian hyperstimulation syndrome: A prospective randomized controlled study. *Fertil Steril.* 2008;89(1):84–91.
 20. Fauzer BC, de Jong D, Olivennes F, et al. Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for in vitro fertilization. *J Clinic Endocrinol Metabol.* 2002;87(2):709–15.
 21. Griffin D, Benadiva C, Kummer N, et al. Dual trigger of oocyte maturation with gonadotropin-releasing hormone agonist and low-dose human chorionic gonadotropin to optimize live birth rates in high responders. *Fertil Steril.* 2012;97(6):1316–20.
 22. Humaidan P. Luteal phase rescue in high-risk OHSS patients by GnRHa triggering in combination with low-dose HCG: A pilot study. *Reprod Biomed Online.* 2009;18(5):630–4.
 23. Humaidan P, Bungum L, Bungum M, et al. Rescue of corpus luteum function with peri-ovulatory HCG supplementation in IVF/ICSI GnRH antagonist cycles in which ovulation was triggered with a GnRH agonist: A pilot study. *Reprod Biomed Online.* 2006;13(2):173–8.
 24. Humaidan P, Bredkjaer E, Westergaard H LG, et al. 1,500 IU human chorionic gonadotropin administered at oocyte retrieval rescues the luteal phase when gonadotropin-releasing hormone agonist is used for ovulation induction: A prospective, randomized, controlled study. *Fertil Steril.* 2010;93(3):847–54.
 25. Humaidan P, Polyzos NP, Alsbjerg B, et al. GnRHa trigger and individualized luteal phase hCG support according to ovarian response to stimulation: Two prospective randomized controlled multi-centre studies in IVF patients. *Hum Reprod.* 2013;28(9):2511–21.
 26. Kol S, Humaidan P, Itskovitz-Eldor J. GnRH agonist ovulation trigger and hCG-based, progesterone-free luteal support: A proof of concept study. *Hum Reprod.* 2011;26(10):2874–7.
 27. Melo M, Busso CE, Bellver J, et al. GnRH agonist versus recombinant HCG in an oocyte donation programme: A randomized, prospective, controlled, assessor-blind study. *Reprod Biomed Online.* 2009;19(4):486–92.
 28. Papanikolaou EG, Verpoest W, Fatemi H, et al. A novel method of luteal supplementation with recombinant luteinizing hormone when A gonadotropin-releasing hormone agonist is used instead of human chorionic gonadotropin for ovulation triggering: A randomized prospective proof of concept study. *Fertil Steril.* 2011;95(3):1174–7.
 29. Shapiro BS, Daneshmand ST, Restrepo H, et al. Efficacy of induced luteinizing hormone surge after “trigger” with gonadotropin-releasing hormone agonist. *Fertil Steril.* 2011;95(2):826–8.
 30. Shapiro BS, Daneshmand ST, Garner FC, et al. Embryo cryopreservation rescues cycles with premature luteinization. *Fertil Steril.* 2010;93(2):636–41.
 31. Oktay K, Turkcuoglu I, Rodriguez-Wallberg KA. GnRH agonist trigger for women with breast cancer undergoing fertility preservation by aromatase inhibitor/FSH stimulation. *Reprod Biomed Online.* 2010;20(6):783–8.
 32. Reddy J, Turan V, Bedoschi G, et al. Triggering final oocyte maturation with gonadotropin-releasing hormone agonist (GnRHa) versus human chorionic gonadotropin (hCG) in breast cancer patients undergoing fertility preservation: An extended experience. *J Assist Reprod Genet.* 2014;31(7):927–32.
 33. Meyer L, Murphy LA, Gumer A, et al. Risk factors for a suboptimal response to gonadotropin-releasing hormone agonist trigger during in vitro fertilization cycles. *Fertil Steril.* 2015;104(3):637–42.
 34. Nakano R, Mizuno T, Kotsuji F, et al. “Triggering” of ovulation after infusion of synthetic luteinizing hormone releasing factor (LRF). *Acta Obstetricia Et Gynecologica Scandinavica.* 1973;52(3):269–72.
 35. Yding Andersen C. Effect of FSH and its different isoforms on maturation of oocytes from pre-ovulatory follicles. *Reprod Biomed Online.* 2002;5(3):232–9.
 36. Zelinski-Wooten MB, Hutchison JS, Hess DL, et al. Follicle stimulating hormone alone supports follicle growth and oocyte development in gonadotrophin-releasing hormone antagonist-treated monkeys. *Hum Reprod.* 1995;10(7):1658–66.
 37. Eppig JJ. FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature.* 1979;281(5731):483–4.
 38. Richards JS, Jahnsen T, Hedin L, et al. Ovarian follicular development: From physiology to molecular biology. *Recent Prog Horm Res.* 1987;43:231–76.
 39. Hoff JD, Quigley ME, Yen SS. Hormonal dynamics at midcycle: A reevaluation. *J Clinic Endocrinol Metabol.* 1983;57(4):792–6.
 40. Chandrasekher YA, Brenner RM, Molskness TA, et al. Titrating luteinizing hormone surge requirements for ovulatory changes in primate follicles. II. Progesterone receptor expression in luteinizing granulosa cells. *J Clinic Endocrinol Metabol.* 1991;133(3):584–9.
 41. Yding Andersen C, Westergaard LG, Figenschau Y, et al. Endocrine composition of follicular fluid comparing human chorionic gonadotrophin to a gonadotrophin-releasing hormone agonist for ovulation induction. *Hum Reprod.* 1993;8(6):840–3.
 42. Andersen CY, Humaidan P, Ejdrup HB, et al. Hormonal characteristics of follicular fluid from women receiving either GnRH agonist or hCG for ovulation induction. *Hum Reprod.* 2006;21(8):2126–30.
 43. Park JY, Su YQ, Ariga M, et al. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science.* 2004;303(5658):682–4.
 44. Humaidan P, Westergaard LG, Mikkelsen AL, Fukuda M, Yding AC. Levels of the EGF-like peptide amphiregulin are significantly reduced in follicular fluid after GnRHa triggering of final oocyte maturation. *Hum Reprod.* 2009;24(O–284).

45. Cerrillo M, Rodriguez S, Mayoral M, et al. Differential regulation of VEGF after final oocyte maturation with GnRH agonist versus hCG: A rationale for OHSS reduction. *Fertil Steril.* 2009;91(4 Suppl):1526–8.
46. Villasante A, Pacheco A, Ruiz A, et al. Vascular endothelial cadherin regulates vascular permeability: Implications for ovarian hyperstimulation syndrome. *J Clinic Endocrinol Metabol.* 2007;92(1):314–21.
47. Cerrillo M, Pacheco A, Rodriguez S, et al. Effect of GnRH agonist and hCG treatment on VEGF, angiopoietin-2, and VE-cadherin: Trying to explain the link to ovarian hyperstimulation syndrome. *Fertil Steril.* 2011;95(8):2517–9.
48. Miller I, Chuderland D, Ron-El R, et al. GnRH agonist triggering modulates PEDF to VEGF ratio inversely to hCG in granulosa cells. *J Clinic Endocrinol Metabol* 2015;100(11):E1428–36.
49. Engmann L, Romak J, Nulsen J, et al. In vitro viability and secretory capacity of human luteinized granulosa cells after gonadotropin-releasing hormone agonist trigger of oocyte maturation. *Fertil Steril.* 2011;96(1):198–202.
50. Ling LP, Phoon JW, Lau MS, et al. GnRH agonist trigger and ovarian hyperstimulation syndrome: Relook at ‘freeze-all strategy’. *Reprod Biomed Online.* 2014;29(3):392–4.
51. Castillo JC, Dolz M, Bienvenido E, et al. Cycles triggered with GnRH agonist: Exploring low-dose HCG for luteal support. *Reprod Biomed Online.* 2010;20(2):175–81.
52. Radesic B, Tremellen K. Oocyte maturation employing a GnRH agonist in combination with low-dose hCG luteal rescue minimizes the severity of ovarian hyperstimulation syndrome while maintaining excellent pregnancy rates. *Hum Reprod.* 2011;26(12):3437–42.
53. Parneix I, Emperaire JC, Ruffie A, et al. [Comparison of different protocols of ovulation induction, by GnRH agonists and chorionic gonadotropin]. *Gynecol Obstetr Fertil.* 2001;29(2):100–5.
54. Kummer NE, Feinn RS, Griffin DW, et al. Predicting successful induction of oocyte maturation after gonadotropin-releasing hormone agonist (GnRHa) trigger. *Hum Reprod.* 2013;28(1):152–9.
55. Babayof R, Margalioth EJ, Huleihel M, et al. Serum inhibin a, VEGF and TNFalpha levels after triggering oocyte maturation with GnRH agonist compared with HCG in women with polycystic ovaries undergoing IVF treatment: a prospective randomized trial. *Hum Reprod.* 2006;21(5):1260–5.
56. Imbar T, Kol S, Lossos F, et al. Reproductive outcome of fresh or frozen-thawed embryo transfer is similar in high-risk patients for ovarian hyperstimulation syndrome using GnRH agonist for final oocyte maturation and intensive luteal support. *Hum Reprod.* 2012;27(3):753–9.
57. Ngoc Lan Vuong T, Tuong Ho M, Duc Ha T, et al. Gonadotropin-releasing hormone agonist trigger in oocyte donors co-treated with A gonadotropin-releasing hormone antagonist: A dose-finding study. *Fertil Steril.* 2016 Feb;105(2):356–63.
58. Lainas GT, Lainas TG, Sfontouris, et al. Is oocyte maturation rate associated with triptorelin dose used for triggering final oocyte maturation in patients at high risk for severe ovarian hyperstimulation syndrome? *Hum Reprod.* 2019;34(9):1770–7.
59. Lainas GT, Lainas TG, Sfontouris IA, et al. Association between body mass index and oocyte maturation in patients triggered with GnRH agonist who are at high risk for severe ovarian hyperstimulation syndrome: An observational cohort study. *Reprod Biomed Online.* 2020;40(1):168–75.
60. Buckett WM, Bentick B, Shaw RW. Induction of the endogenous gonadotrophin surge for oocyte maturation with intra-nasal gonadotrophin-releasing hormone analogue (buserelin): Effective minimal dose. *Hum Reprod.* 1998;13(4):811–4.
61. Horowitz E, Mizrachi Y, Farhi J, et al. Does the interval between the last GnRH antagonist dose and the GnRH agonist trigger affect oocyte recovery and maturation rates? *Reprod Biomed Online.* 2020;41(5):917–24.
62. Oktay K, Buyuk E, Rodriguez-Wallberg KA, et al. In vitro maturation improves oocyte or embryo cryopreservation outcome in breast cancer patients undergoing ovarian stimulation for fertility preservation. *Reprod Biomed Online.* 2010;20(5):634–8.
63. Hernandez ER, Gomez-Palomares JL, Ricciarelli E. No room for cancellation, coasting, or ovarian hyperstimulation syndrome in oocyte donation cycles. *Fertil Steril.* 2009;91(4 Suppl):1358–61.
64. Bodri D, Guillen JJ, Galindo A, et al. Triggering with human chorionic gonadotropin or a gonadotropin-releasing hormone agonist in gonadotropin-releasing hormone antagonist-treated oocyte donor cycles: Findings of a large retrospective cohort study. *Fertil Steril.* 2009;91(2):365–71.
65. Yilmaz N, Ceren MU, Ugurlu EN, et al. GnRH agonist versus HCG triggering in different IVF/ICSI cycles of same patients: A retrospective study. *J Obstetr Gynaecol.* 2020;40(6):837–42.
66. Maslow BSL, Guarascia M, Stefanacci C, et al. The use of GnRH-agonist trigger for the final maturation of oocytes in normal and low responders undergoing planned oocyte cryopreservation. *Hum Reprod.* 2020;35(5):1054–60.
67. Acevedo B, Gomez-Palomares JL, Ricciarelli E, et al. Triggering ovulation with gonadotropin-releasing hormone agonists does not compromise embryo implantation rates. *Fertil Steril.* 2006;86(6):1682–7.
68. Sismanoglu A, Tekin HI, Erden HF, et al. Ovulation triggering with GnRH agonist vs. hCG in the same egg donor population undergoing donor oocyte cycles with GnRH antagonist: A prospective randomized cross-over trial. *J Assist Reprod Genet.* 2009;26(5):251–6.
69. Sahin S, Ozay A, Ergin E, et al. The risk of ectopic pregnancy following GnRH agonist triggering compared with hCG triggering in GnRH antagonist IVF cycles. *Arch Gynecol Obstetr.* 2015;291(1):185–91.
70. Christopoulos G, Vlismas A, Carby A, et al. GnRH agonist trigger with intensive luteal phase support vs. human chorionic gonadotropin trigger in high responders: An observational study reporting pregnancy outcomes And incidence of ovarian hyperstimulation syndrome. *Hum Fertil.* 2016;19(3):199–206.
71. Gurbuz AS, Gode F, Uzman MS, et al. GnRH agonist triggering affects the kinetics of embryo development: A comparative study. *J Ovarian Res.* 2016;9(1):1–6.
72. Chen SL, Ye DS, Chen X, et al. Circulating luteinizing hormone level after triggering oocyte maturation with GnRH agonist may predict oocyte yield in flexible GnRH antagonist protocol. *Hum Reprod.* 2012;27(5):1351–6.
73. Herman HG, Horowitz E, Mizrachi Y, Farhi J, Raziel A, Weissman A. Prediction, assessment, and management of suboptimal GnRH agonist trigger: A systematic review. *J Assist Reprod Genet.* 2022;39(2):291–303.
74. Popovic-Todorovic B, Santos-Ribeiro S, Drakopoulos P, et al. Predicting suboptimal oocyte yield following GnRH agonist trigger by measuring serum LH at the start of ovarian stimulation. *Hum Reprod.* 2019;34(10):2027–35.
75. Chang FE, Beall SA, Cox JM, et al. Assessing the adequacy of gonadotropin-releasing hormone agonist leuprolide to trigger oocyte maturation and management of inadequate response. *Fertil Steril.* 2016;106(5):1093–1100.
76. Asada Y, Itoi F, Honnma H, et al. Failure of GnRH agonist-triggered oocyte maturation: Its cause and management. *J Assist Reprod Genet.* 2013;30(4):581–5.
77. Castillo JC, Garcia-Velasco J, Humaidan P. Empty follicle syndrome after GnRHa triggering versus hCG triggering in COS. *J Assist Reprod Genet.* 2012;29(3):249–53.
78. Baum M, Machtlinger R, Yerushalmi GM, et al. Recurrence of empty follicle syndrome with stimulated IVF cycles. *Gynecol Endocrinol.* 2012;28(4):293–5.
79. Ben-Shlomo I, Schiff E, Levran D, et al. Failure of oocyte retrieval during in vitro fertilization: A sporadic event rather than A syndrome. *Fertil Steril.* 1991;55(2):324–7.

80. Mesen TB, Yu B, Richter KS, et al. The prevalence of genuine empty follicle syndrome. *Fertil Steril.* 2011;96(6):1375–7.
81. Quintans CJ, Donaldson MJ, Blanco LA, et al. Empty follicle syndrome due to human errors: Its occurrence in an in-vitro fertilization programme. *Hum Reprod.* 1998;13(10):2703–5.
82. Zegers-Hochschild F, Fernandez E, Mackenna A, et al. The empty follicle syndrome: A pharmaceutical industry syndrome. *Hum Reprod.* 1995;10(9):2262–5.
83. Lu X, Hong Q, Sun LH, et al. Dual trigger for final oocyte maturation improves the oocyte retrieval rate of suboptimal responders to gonadotropin-releasing hormone agonist. *Fertil Steril.* 2016;106(6):1356–62.
84. Worldwide I. Survey on vitrification, GnRH trigger and differed embryo transfer. 2013. <http://www.ivf-worldwide.com/survey/vitrification,-gnrh-trigger-and-differed-et/results-vitrification,-gnrh-trigger-and-differed-et.html>.
85. Griffin D, Feinn R, Engmann L, et al. Dual trigger with gonadotropin-releasing hormone agonist and standard dose human chorionic gonadotropin to improve oocyte maturity rates. *Fertil Steril.* 2014;102(2):405–9.
86. Fabris AM, Cruz M, Legidos V, et al. Dual triggering with gonadotropin-releasing hormone agonist and standard dose human chorionic gonadotropin in patients with a high immature oocyte rate. *Reprod Sci.* 2017;24(8):1221–25.
87. Ali SS, Elsenosy E, Sayed GH, et al. Dual trigger using recombinant HCG and gonadotropin-releasing hormone agonist improve oocyte maturity and embryo grading for normal responders in GnRH antagonist cycles: Randomized controlled trial. *J Gynecol Obstetr Hum Reprod.* 2020;49(5):101728.
88. Maged AM, Ragab MA, Shohayeb A, et al. Comparative study between single versus dual trigger for poor responders in GnRH-antagonist ICSI cycles: A randomized controlled study. *Int J Gynecol Obstetr.* 2021;152(3):395–400.
89. Zhou X, Guo P, Chen X, et al. Comparison of dual trigger with combination GnRH agonist and hCG versus hCG alone trigger of oocyte maturation for normal ovarian responders. *Int J Gynecol Obstetr.* 2018;141(3):327–31.
90. Haas J, Zilberman E, Nahum R, et al. Does double trigger (GnRH-agonist + hCG) improve outcome in poor responders undergoing IVF-ET cycle? A pilot study. *Gynecol Endocrinol.* 2019;35(7):628–30.
91. Chern CU, Li JY, Tsui KH, et al. Dual-trigger improves the outcomes of in vitro fertilization cycles in older patients with diminished ovarian reserve: A retrospective cohort study. *PLoS One.* 2020;15(7):e0235707.
92. Hu KL, Wang S, Ye X, et al. GnRH agonist and hCG (dual trigger) versus hCG trigger for follicular maturation: A systematic review and meta-analysis of randomized trials. *Reprod Biol Endocrinol.* 2021;19(78):1–10.
93. Haas J, Bassil R, Samara, et al. GnRH agonist and hCG (dual trigger) versus hCG trigger for final follicular maturation: A double-blinded, randomized controlled study. *Hum Reprod.* 2020;35(7):1648–54.
94. Zilberman E, Haas J, Dar S, et al. Co-administration of GnRH-agonist and hCG, for final oocyte maturation (double trigger), in patients with low proportion of mature oocytes. *Gynecol Endocrinol.* 2015;31(2):145–7.
95. Iliodromiti S, Lan VT, Tuong HM, et al. Impact of GnRH agonist triggering and intensive luteal steroid support on live-birth rates and ovarian hyperstimulation syndrome: A retrospective cohort study. *J Ovarian Res.* 2013;6(1):93.
96. McCracken JA, Custer EE, Lamsa JC. Luteolysis: A neuroendocrine-mediated event. *Physiol Rev.* 1999;79(2):263–323.
97. Wang TH, Horng SG, Chang CL, et al. Human chorionic gonadotropin-induced ovarian hyperstimulation syndrome is associated with up-regulation of vascular endothelial growth factor. *J Clinic Endocrinol Metabol.* 2002;87(7):3300–8.
98. Sugino N, Kashida S, Takiguchi S, et al. Expression of vascular endothelial growth factor and its receptors in the human corpus luteum during the menstrual cycle and in early pregnancy. *J Clinic Endocrinol Metabol.* 2000;85(10):3919–24.
99. Rao CV. Multiple novel roles of luteinizing hormone. *Fertil Steril.* 2001;76(6):1097–100.
100. Tannus S, Burke Y, McCartney CR, et al. GnRH-agonist triggering for final oocyte maturation in GnRH-antagonist IVF cycles induces decreased LH pulse rate and amplitude in early luteal phase: A possible luteolysis mechanism. *Gynecol Endocrinol.* 2017;33(9):741–5.
101. Kaye L, Griffin D, Thorne J, et al. Independent serum markers of corpora lutea function after gonadotropin-releasing hormone agonist trigger and adjuvant low dose human chorionic gonadotropin in in vitro fertilization. *Fertil Steril.* 2019;112(3):534–44.
102. Zelinski-Wooten MB, Lanzendorf SE, Wolf DP, et al. Titrating luteinizing hormone surge requirements for ovulatory changes in primate follicles. I. Oocyte maturation and corpus luteum function. *J Clinic Endocrinol Metabol.* 1991;173(3):577–83.
103. Zelinski-Wooten MB, Hutchison JS, Chandrasekher YA, et al. Administration of human luteinizing hormone (hLH) to macaques after follicular development: Further titration of LH surge requirements for ovulatory changes in primate follicles. *J Clinic Endocrinol Metabol.* 1992;75(2):502–7.
104. Kol S. Luteolysis induced by a gonadotropin-releasing hormone agonist is the key to prevention of ovarian hyperstimulation syndrome. *Fertil Steril.* 2004;81(1):1–5.
105. Fauser BC, Devroey P. Reproductive biology and IVF: Ovarian stimulation and luteal phase consequences. *Trends Endocrinol Metab.* 2003;14(5):236–42.
106. Nevo O, Eldar-Geva T, Kol S, et al. Lower levels of inhibin a and pro-alphaC during the luteal phase after triggering oocyte maturation with a gonadotropin-releasing hormone agonist versus human chorionic gonadotropin. *Fertil Steril.* 2003;79(5):1123–8.
107. Bermejo A, Cerrillo M, Ruiz-Alonso M, et al. Impact of final oocyte maturation using gonadotropin-releasing hormone agonist triggering and different luteal support protocols on endometrial gene expression. *Fertil Steril.* 2014;101(1):138–46 e3.
108. Humaidan P, Van Vaerenbergh I, Bourgoin C, et al. Endometrial gene expression in the early luteal phase is impacted by mode of triggering final oocyte maturation in recFSH stimulated and GnRH antagonist co-treated IVF cycles. *Hum Reprod.* 2012;27(11):3259–72.
109. McCartney CR, Eagleson CA, Marshall JC. Regulation of gonadotropin secretion: Implications for polycystic ovary syndrome. *Semin Reprod Med.* 2002;20(4):317–26.
110. Griesinger G, Diedrich K, Devroey P, et al. GnRH agonist for triggering final oocyte maturation in the GnRH antagonist ovarian hyperstimulation protocol: A systematic review and meta-analysis. *Hum Reprod Update.* 2006;12(2):159–68.
111. Engmann L, Siano L, Schmidt D, et al. GnRH agonist to induce oocyte maturation during IVF in patients at high risk of OHSS. *Reprod Biomed Online.* 2006;13(5):639–44.
112. Iliodromiti S, Blockeel C, Tremellen KP, et al. Consistent high clinical pregnancy rates and low ovarian hyperstimulation syndrome rates in high-risk patients after GnRH agonist triggering and modified luteal support: A retrospective multicentre study. *Hum Reprod.* 2013;28(9):2529–36.
113. Shapiro BS, Daneshmand ST, Garner FC, et al. Gonadotropin-releasing hormone agonist combined with a reduced dose of human chorionic gonadotropin for final oocyte maturation in fresh autologous cycles of in vitro fertilization. *Fertil Steril.* 2008;90(1):231–3.
114. Shapiro BS, Daneshmand ST, Garner FC, et al. Comparison of “triggers” using leuprolide acetate alone or in combination with low-dose human chorionic gonadotropin. *Fertil Steril.* 2011;95(8):2715–7.

115. Humaidan P, Thomsen LH, Alsbjerg B. GnRH-a trigger and modified luteal support with one bolus of hCG should be used with caution in extreme responder patients. *Hum Reprod.* 2013;28(9):2593–4.
116. Engmann LL, Maslow BS, Kaye LA, et al. Low dose human chorionic gonadotropin administration at the time of gonadotropin-releasing-hormone agonist trigger versus 35 h later in women at high risk of developing ovarian hyperstimulation syndrome - a prospective randomized double-blind clinical trial. *J Ovarian Res.* 2019;12(1):1–9.
117. Fatemi HM. The luteal phase after 3 decades of IVF: What do we know? *Reprod Biomed Online.* 2009;19(Suppl 4):4331.
118. Mitwally MF, Diamond MP, Abuzeid M. Vaginal micronized progesterone versus intramuscular progesterone for luteal support in women undergoing in vitro fertilization-embryo transfer. *Fertil Steril.* 2010;93(2):554–69.
119. Karacan M, Erdem E, Usta A, et al. Gonadotropin-releasing hormone agonist triggering with concomitant administration of low doses of human chorionic gonadotropin or a freeze-all strategy in high responders. *Saudi Med J.* 2017;38(6):586–91.
120. Elgindy EA, Sibai H, Mostafa MI, et al. Towards an optimal luteal support modality in agonist triggered cycles: A randomized clinical trial. *Hum Reprod.* 2018;33(6):1079–86.
121. Seyhan A, Ata B, Polat M, et al. Severe early ovarian hyperstimulation syndrome following GnRH agonist trigger with the addition of 1500 IU hCG. *Hum Reprod.* 2013;28(9):2522–8.
122. Vanetik S, Segal L, Breizman T, et al. Day two post retrieval 1500 IU hCG bolus, progesterone-free luteal support post GnRH agonist trigger—a proof of concept study. *Gynecol Endocrinol.* 2018;34(2):132–5.
123. Kol S, Segal L. GnRH agonist triggering followed by 1500 IU of HCG 48 h after oocyte retrieval for luteal phase support. *Reprod Biomed Online.* 2020;41(5):854–8.
124. Elbaek HO, AB, Laursen R, Povlsen BB, Mikkelsen AT, Andersen CY, Humaidan P. The exogenous progesterone free luteal phase in IVF - exploring a new concept. *Hum Reprod.* 2014;29(i326):502.
125. Andersen CY, Elbaek HO, Alsbjerg B, et al. Daily low-dose hCG stimulation during the luteal phase combined with GnRH-a triggered IVF cycles without exogenous progesterone: A proof of concept trial. *Hum Reprod.* 2015;30(10):2387–95.
126. Fusi FM, Arnoldi M, Bosisio C, et al. Ovulation induction and luteal support with GnRH agonist in patients at high risk for hyperstimulation syndrome. *Gynecol Endocrinol.* 2015;31(9):693–7.
127. Kol S, Breyzman T, Segal L, et al. 'Luteal coasting' after GnRH agonist trigger - individualized, HCG-based, progesterone-free luteal support in 'high responders': A case series. *Reprod Biomed Online.* 2015;31(6):747–751.
128. Lawrenz B, Ruiz F, Engelmann N, et al. Individual luteolysis post GnRH-agonist-trigger in GnRH-antagonist protocols. *Gynecol Endocrinol.* 2017;33(4):261–4.
129. Lawrenz B, Samir S, Melado L, et al. Luteal phase serum progesterone levels after GnRH-agonist trigger - how low is still high enough for an ongoing pregnancy? *Gynecol Endocrinol.* 2018;34(3):195–8.
130. Filicori M, Butler JP, Crowley WF. Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion. *Obstetrical and Gynecological Survey.* 1985;40(4):240–2.
131. Devroey P, Polyzos NP, Blockeel C. An OHSS-free clinic by segmentation of IVF treatment. *Hum Reprod.* 2011;26(10):2593–7.
132. Garcia-Velasco JA. Agonist trigger: What is the Best approach? Agonist trigger with vitrification of oocytes or embryos. *Fertil Steril.* 2012;97(3):527–8.
133. Griesinger G, Berndt H, Schultz L, et al. Cumulative live birth rates after GnRH-agonist triggering of final oocyte maturation in patients at risk of OHSS: A prospective, clinical cohort study. *Eur J Obstetr Gynecol Reprod Biol.* 2010;149(2):190–4.
134. Griesinger G, Schultz L, Bauer T, et al. Ovarian hyperstimulation syndrome prevention by gonadotropin-releasing hormone agonist triggering of final oocyte maturation in A gonadotropin-releasing hormone antagonist protocol in combination with A "freeze-all" strategy: A prospective multicentric study. *Fertil Steril.* 2011;95(6):2029–33, 33 e1.
135. Manzanares MA, Gomez-Palomares JL, Ricciarelli E, et al. Triggering ovulation with gonadotropin-releasing hormone agonist in in vitro fertilization patients with polycystic ovaries does not cause ovarian hyperstimulation syndrome despite very high estradiol levels. *Fertil Steril.* 2010;93(4):1215–9.
136. Makhijani R, Thorne J, Bartels C, et al. Pregnancy outcomes after frozen-thawed single euploid blastocyst transfer following IVF cycles using GnRH agonist or HCG trigger for final oocyte maturation. *J Assist Reprod Genet.* 2020;37(3):611–7.
137. Benadiva C, Engmann L. Luteal phase support after gonadotropin-releasing hormone agonist triggering: Does it still matter? *Fertil Steril.* 2018;109(5):763–7.
138. Kummer N, Benadiva C, Feinn R, et al. Factors that predict the probability of a successful clinical outcome after induction of oocyte maturation with a gonadotropin-releasing hormone agonist. *Fertil Steril.* 2011;96(1):63–8.
139. Bodri D. Low-dose hCG supplementation after GnRH agonist triggering: Don't be too quick on the trigger. *Hum Reprod.* 2013;28(9):2315–7.
140. Humaidan P, Engmann L, Benadiva C. Luteal phase supplementation after gonadotropin-releasing hormone agonist trigger in fresh embryo transfer: The American versus European approaches. *Fertil Steril.* 2015;103(4):879–85.
141. Humaidan P, Kol S, Papanikolaou EG, et al. GnRH agonist for triggering of final oocyte maturation: Time for a change of practice? *Hum Reprod Update.* 2011;17(4):510–24.
142. Youssef MA, Van der Veen F, Al-Inany HG, et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database Syst Rev.* 2014;10:CD008046.
143. Fatemi HM, Popovic-Todorovic B, Humaidan P, et al. Severe ovarian hyperstimulation syndrome after gonadotropin-releasing hormone (GnRH) agonist trigger and "freeze-all" approach in GnRH antagonist protocol. *Fertil Steril.* 2014;101(4):1008–11.
144. O'Neill KE, Senapati S, Maina I, et al. GnRH agonist with low-dose hCG (dual trigger) is associated with higher risk of severe ovarian hyperstimulation syndrome compared to GnRH agonist alone. *J Assist Reprod Genet.* 2016;33(9):1175–84.
145. Thorne J, Loza A, Kaye L, et al. Euploidy rates between cycles triggered with gonadotropin-releasing hormone agonist and human chorionic gonadotropin. *Fertil Steril.* 2019;112(2):258–65.
146. Shapiro BS, Daneshmand ST, Garner FC, et al. Comparison of human chorionic gonadotropin and gonadotropin-releasing hormone agonist for final oocyte maturation in oocyte donor cycles. *Fertil Steril.* 2007;88(1):237–9.
147. Galindo A, Bodri D, Guillen JJ, et al. Triggering with HCG or GnRH agonist in GnRH antagonist treated oocyte donation cycles: A randomised clinical trial. *Gynecol Endocrinol.* 2009;25(1):60–6.
148. Bodri D, Guillen JJ, Trullenque M, et al. Early ovarian hyperstimulation syndrome is completely prevented by gonadotropin-releasing-hormone agonist triggering in high-risk oocyte donor cycles: A prospective, luteal-phase follow-up study. *Fertil Steril.* 2010;93(7):2418–20.
149. Budinetz TH, Mann JS, Griffin DW, et al. Maternal and neonatal outcomes after gonadotropin-releasing hormone agonist trigger for final oocyte maturation in patients undergoing in vitro fertilization. *Fertil Steril.* 2014;102(3):753–8.
150. Garcia-Velasco JA, Motta L, Lopez A, et al. Low-dose human chorionic gonadotropin versus estradiol/progesterone luteal phase support in gonadotropin-releasing hormone agonist-triggered assisted reproductive technique cycles: Understanding a new approach. *Fertil Steril.* 2010;94(7):2820–3.
151. Berkkanoglu M, Coetzee K, Bulut H, et al. Risk of ovarian torsion is reduced in GnRH agonist triggered freeze-all cycles: A retrospective cohort study. *J Obstet Gynaecol.* 2019;39(2):212–7.

152. Castillo JC, M J, Dolz M, Bonilla-Musoles F. Successful pregnancy following dual triggering concept (rhCG & GnRH agonist) in a patient showing repetitive immature oocytes and empty follicle syndrome: Case report. *J Med Cases.* 2013;5: 221–6.
153. Lok F, Pritchard J, Lashen H. Successful treatment of empty follicle syndrome by triggering endogenous LH surge using GnRH agonist in an antagonist down-regulated IVF cycle. *Hum Reprod.* 2003;18(10):2079–81.
154. Pirard X, Donnez J, Loumaye E. GnRH agonist as luteal phase support in assisted reproduction technique cycles: Results of a pilot study. *Hum Reprod.* 2006;21(7):1894–900.
155. Griesinger G, Kolibianakis EM, Papanikolaou EG, Diedrich K, Van Steirteghem A, Devroey P, Ejdrup Bredkjaer H, Humaidan P. Triggering of final oocyte maturation with gonadotropin-releasing hormone agonist or human chorionic gonadotropin. Live birth after frozen-thawed embryo replacement cycles. *Fertil Steril.* 2007;88(3):616–21.

SEGMENTATION OF IN VITRO FERTILIZATION CYCLES

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Introduction

The concept of *in vitro* fertilization (IVF) segmentation, also called “freeze-all,” refers to the strategy of performing oocyte retrieval and embryo transfer in different cycles. Although it was initially introduced as an approach to reduce the risk of ovarian hyperstimulation syndrome (OHSS) [1], it is becoming increasingly prevalent in modern assisted reproduction techniques (ART) for a variety of clinical indications (Figure 45.1). First, the use of pre-implantation genetic testing (PGT) is gaining popularity [2, 3] and often implies deferred frozen embryo transfer (FET) until biopsy results become available. Moreover, the steady increase in oocyte donation cycles [4] and the recognition of oocyte cryopreservation as an established option for fertility preservation [5] also account for widespread use of cycle segmentation. Finally, freeze-all is mandatory in alternative ovarian stimulation protocols such as “random start” or “DuoStim” and is a reasonable approach in some clinical circumstances in which fresh IVF cycles would impair the reproductive outcomes, such as late follicular-phase progesterone elevation or inadequate endometrial development.

This chapter will review the treatment protocols and clinical indications for IVF cycle segmentation.

Segmentation in IVF cycles: How?

Ovarian stimulation in freeze-all cycles

Optimal number of oocytes retrieved

In fresh IVF cycles, 15 oocytes have become the accepted target to maximize the live birth rate (LBR) before significantly increasing the risk of OHSS [6, 7]. However, in segmented cycles, the risk of severe OHSS is virtually eliminated [1], allowing for a safe increase in the target number of oocytes to be retrieved. This has introduced a new, more meaningful outcome measure in ART, the cumulative live birth rate (CLBR), including fresh transfer and all subsequent transfers of frozen-thawed embryos per oocyte retrieval [8]. In fact, large cohort studies have shown an increase in the CLBR, with up to 20 oocytes retrieved [9, 10], or even a continuous increase in CLBR with the number of either fresh [11–13] or vitrified oocytes [14].

Pituitary suppression

Although gonadotropin releasing hormone (GnRH) agonists have been used in IVF since the 1980s, GnRH antagonists have become the predominant strategy for LH suppression in IVF, allowing for a more patient-friendly protocol and a significant reduction in the risk of OHSS with comparable LBR [15–17] as well as CLBR [18]. However, the need for subcutaneous administration, the cost, and the specific side effects of GnRH analogues led to a search for more convenient therapeutic options.

The inhibitory effect of progesterone on gonadotropin secretion has been recognized for decades and is the cornerstone of

hormonal contraception. However, only recently progestins have been introduced as an alternative to prevent premature LH surges during ovarian stimulation [19], and they have proven to be an effective strategy for pituitary suppression during IVF in terms of oocyte yield and pregnancy outcomes with either medroxyprogesterone acetate [19–23], dydrogesterone [24], micronized progesterone [25, 26], or desogestrel [27, 28]. Despite the higher convenience and lower costs of progestin-primed ovarian stimulation (PPOS) protocols when compared to the conventional GnRH analogues, due to progesterone-induced secretory changes of the endometrium, a freeze-all approach is mandatory in these patients [29].

Final oocyte maturation triggering

For many decades, human chorionic gonadotropin (hCG) has been the standard of care for final oocyte maturation, substituting the endogenous mid-cycle LH surge. However, considering its longer half-life, lasting up to six days following administration, the risk of OHSS cannot be disregarded [1]. Therefore, the GnRH agonist (GnRHa) trigger has emerged as an alternative strategy, virtually eliminating the risk of OHSS [1]. In fact, the combination of GnRH antagonist protocol with GnRHa trigger is currently the standard of care in freeze-all cycles [30]. When compared with the traditional HCG trigger, GnRHa presents a shorter, more physiologic follicular stimulating hormone (FSH) and luteinizing hormone (LH) peak, terminating 24 hours after its onset [31, 32]. Although this luteolytic effect has been reported to impair pregnancy outcomes in fresh autologous IVF cycles [33], a similar number of oocytes retrieved and similar pregnancy outcomes have been reported with GnRHa and hCG in FET cycles [34] and in oocyte donation cycles [33]. Corroborating these findings, improved oocyte and embryo quality have been reported following GnRHa triggering when compared to hCG trigger [35, 36]. Moreover, OHSS is an extremely rare event in patients undergoing an antagonist protocol with GnRHa trigger and a freeze-all approach [37, 38], supporting its added value in improving patient safety.

More recently, the concomitant administration of both GnRHa and a bolus of HCG prior to oocyte retrieval (dual trigger) has been proposed as a new strategy for final follicular maturation. By adding the more physiological LH and FSH peak provided by GnRHa to the longer luteal phase support and amplified steroidogenic response provided by hCG, the dual trigger aims to improve oocyte and embryo quality, as well as pregnancy outcomes [39]. With this approach, several studies have reported an increase in the number of MII oocytes retrieved, as well as in the number of good quality embryos and improved pregnancy outcomes in different subpopulations of infertile patients undergoing fresh IVF cycles [40–43]. Up to date, only one retrospective cohort study has analysed the impact of the dual trigger in FET cycles [44]. The authors analysed 4438 freeze-all IVF/intracytoplasmic sperm injection (ICSI) cycles and reported improved LBR (31.7%

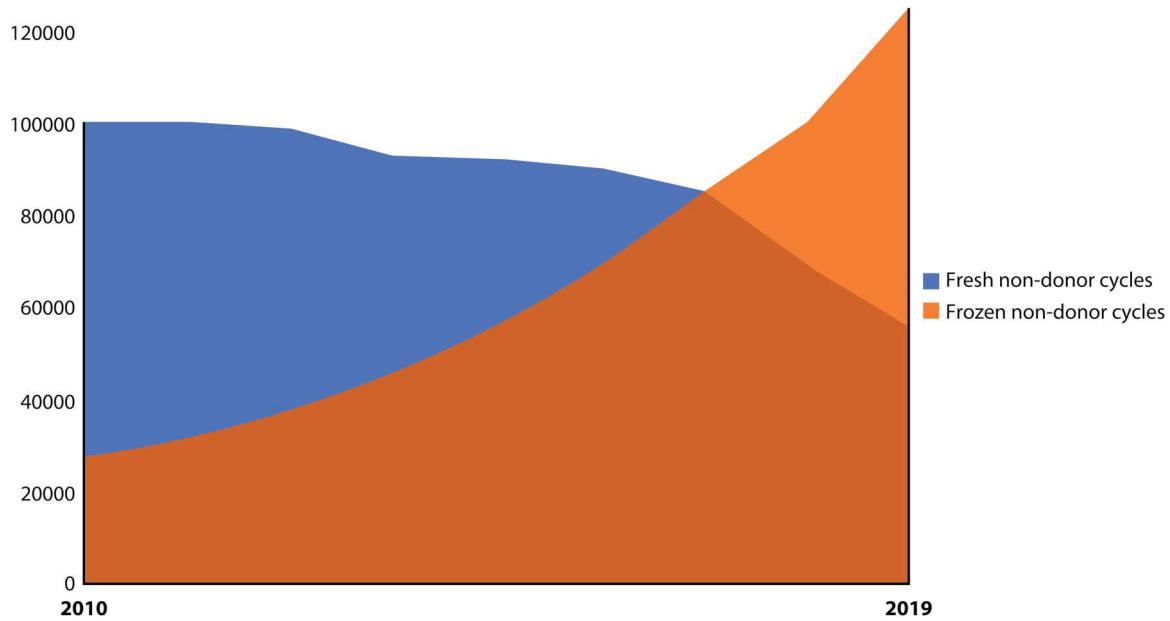


FIGURE 45.1 Number of non-donor ART cycles. (Data from Centers for Disease Control and Prevention, ART success rates, 2010–2019; available at <https://www.cdc.gov/art/reports/archive.html>)

vs 26.6%, $P < 0.001$; OR 0.783 (0.709–0.864) and CLBR (50.5% vs 44.3%, $P < 0.001$; OR 0.781; 95% CI 0.688–10.886) following dual trigger when compared to hCG [44].

To sum up, the GnRH antagonist protocol with GnRHa trigger is currently the gold standard for patients undergoing a freeze-all approach [30]. More evidence on the use of the dual trigger in segmented cycles is needed before adopting this strategy in daily clinical practice. In this regard, the currently undergoing randomized controlled trial Dual Trigger for Elective Fertility Preservation (DUAL-T) (<https://clinicaltrials.gov/ct2/show/NCT04992468>) might shed some light on this subject. By comparing the dual trigger with the GnRHa trigger in patients undergoing elective (non-medical) oocyte cryopreservation, the results of this trial are expected to clarify whether the dual trigger should be the preferred approach in all patients undergoing IVF segmentation.

Cryopreservation of oocytes or embryos

Oocyte and embryo cryopreservation is nowadays considered a key element in modern ART. Since the introduction of cryobiology to ART, two main protocols have been adopted: first, slow freezing, and, more recently, vitrification. During slow freezing, cells are exposed to a slow decrease in temperature until a temperature of -30°C has been reached and then reduced rapidly until -150°C before being added to liquid nitrogen for storage [45]. On the other hand, with vitrification, the immediate exposure to liquid nitrogen induces a quick temperature reduction, allowing for the occurrence of solidification without ice formation [45].

The superiority of oocyte vitrification compared to slow freezing has been demonstrated in a recent meta-analysis in terms of clinical pregnancy rate (CPR) (RR 2.81, 95% CI 1.05–7.51; 1 RCT; n = 78) and cryo-survival (RR 1.23; 95% CI 1.02–1.49; 3 RCT; n = 1181) [46]. Also, embryo vitrification resulted in a significantly higher CPR per transfer (RR 1.51; 95% CI 1.03–2.23; 3 RCT; n = 488) and

cryo-survival (RR 1.59; 95% CI 1.30–1.93; 7 RCT; n = 3615) when compared to slow freezing [46].

The proven efficacy and safety of the vitrification technique has contributed to increase the clinicians' confidence in adopting a freeze-all approach. In fact, accumulating evidence demonstrates non-inferior pregnancy rates and LBR when vitrified and warmed oocytes and embryos are compared with fresh cycles [47–50]. Together with a shift from transfer of cleavage stage to blastocyst stage embryos, the generalized use of vitrification has also contributed to an increase in the CLBR following ART during the last decade [51].

Segmentation in IVF cycles: When?

Risk of OHSS

OHSS is a serious, mainly iatrogenic, complication of controlled ovarian stimulation. A recognized primary strategy to minimize the risk of OHSS is performing ovarian stimulation in a GnRH antagonist protocol, with GnRHa trigger and a freeze-all strategy [34, 52]. In fact, two Cochrane meta-analyses found a lower incidence of OHSS following GnRHa trigger when compared to the hCG trigger in donor-recipient cycles (OR 0.05, 95% CI 0.01–0.28; 3 RCTs, 374 women, $I^2 = 0\%$) [33], as well as a lower incidence of OHSS with the freeze-all strategy when compared to fresh IVF/ICSI cycles (OR 0.26, 95% CI 0.17–0.39; 6 RCTs, 4478 women; $I^2 = 0\%$) [53]. Therefore, IVF segmentation in patients at risk of OHSS has transformed a potentially critical complication into an anecdotal event.

Avoidance of embryo–endometrium desynchrony

Slowly growing embryos

Advances in embryo culture media and co-culture techniques have led to a shift to the practice of blastocyst-stage embryo transfer instead of the conventional cleavage-stage embryo transfer, with improved LBR following IVF/ICSI cycles [54, 55].

Although *in vitro* cultured embryos usually reach the blastocyst stage five days after fertilization, slower embryos can still achieve successful blastulation on day 6 or even later. A recent large-scale retrospective cohort study has shown that, while slowly blastulating D5 or D6 embryos present a lower implantation rate in fresh embryo transfer (ET) cycles when compared to their normally blastulating counterparts, no difference was observed when frozen embryo transfer (FET) was performed [56]. These results suggest that slowly growing embryos are equivalent to normally blastulating embryos in terms of reproductive potential when an elective FET is performed, accounting for embryo–endometrium synchrony.

Traditionally, extended culture to the blastocyst stage has been considered up to day 5 or 6 of embryo culture [57, 58]. However, more recently, day 7 cryopreservation has been introduced in clinical practice. Although the mechanisms for the delayed embryo development are yet to be elucidated, day 7 blastocysts have proven to be suitable for biopsy, with acceptable euploidy rates [59, 60] and clinically relevant outcomes in terms of pregnancy rates and LBR [59, 61–63]. Therefore, day 7 blastocysts are now proposed as good candidates for embryo transfer when no day 5 or day 6 embryos are available [64]. However, in order to avoid embryo–endometrium asynchrony, cryopreservation of day 7 embryos and an elective FET in a subsequent cycle is recommended.

Pre-implantation genetic testing

Pre-implantation genetic testing (PGT) is increasingly being used in ART. According to the latest American Society of Reproductive Medicine (ASRM) report, between 39% and 50% of embryo transfers included at least one embryo with PGT [65]. PGT was initially performed in first polar bodies (PB) and cleavage-stage embryos [66, 67], allowing for embryo transfer in the same cycle. However, the low capture rate and low accuracy of PB biopsy and the 40% decrease in implantation rate following blastomere biopsy [68, 69] have led to a shift to trophectoderm biopsy at the blastocyst stage. The possibility of trophectoderm biopsy and fresh day 6 embryo transfer has recently been analysed in a randomized controlled trial (RCT) [70]. However, the authors concluded that significantly higher ongoing pregnancy rates and LBR were observed in the FET group when compared to the day 6 fresh ET group. Therefore, PGT with trophoblast biopsy should be considered as an indication for cycle segmentation.

Late follicular-phase progesterone rise

Late follicular-phase progesterone elevation (PE) has been associated with impaired pregnancy outcomes in fresh IVF/ICSI cycles [71–74]. This supra-physiological PE appears to be driven by the number of follicles, the dose of gonadotropins and their effect on the granulosa cells, as well as the effect of LH stimulation on the theca cells [75, 76]. This is in line with reports of higher gonadotropin dose, oestradiol levels, and number of oocytes retrieved in patients with PE [71, 73, 77–79].

This negative effect seems to be explained by an accelerated endometrial maturation, induction of an abnormal gene expression profile and abnormal expression of implantation-regulating proteins, resulting in an impaired endometrial receptivity [80–83]. Of interest, several studies have shown that performing FET cycles or oocyte-recipient cycles mitigates this effect, reinforcing the endometrial impact of PE [71, 78, 84–87]. In line with these findings, two recent studies have reported similar embryo euploidy rates and blastulation rates [85, 88], as well as similar

CLBR [85] in cycles with and without PE, further corroborating a mainly endometrial effect of the supra-physiological hormone levels, and defying previous reports of a potential effect at the oocyte/embryo level [89–91].

Despite the fact that the exact threshold beyond which progesterone levels impair reproductive outcomes is still a matter of controversy, segmentation of IVF cycles might be of value in these patients to overcome the adverse endometrial effect and restore embryo–endometrium synchrony.

Non-conventional protocols of ovarian stimulation

The documentation that follicular development occurs in a wave-like fashion provided the grounds for the implementation of new ovarian stimulation regimens such as random start stimulation, in which stimulation is initiated at any time during the menstrual cycle, and dual stimulation (DuoStim), in which two stimulations are performed in the same cycle [92–94]. In both approaches, freeze-all is mandatory to avoid embryo–endometrium asynchrony.

Random start stimulation has been introduced in the context of urgent fertility preservation [94]. However, in the last few years, its application has expanded beyond this setting. A longer duration of stimulation and higher gonadotropin consumption have been reported in late follicular phase or luteal phase stimulation when compared to early follicular phase stimulation [95–98]. However, the available evidence is reassuring regarding the number of mature oocytes retrieved, available embryos, and pregnancy outcomes [96–99]. Also, no difference has been reported in terms of perinatal outcomes, although larger sample sizes and long-term follow-up studies are needed to confirm these findings [100, 101].

The double stimulation during the follicular and luteal phase in the same cycle was introduced in patients with poor ovarian response (POR) to maximize the number of oocytes retrieved in a shorter timeframe [102]. This approach has shown to maximize the number of oocytes retrieved and, therefore, the number of available embryos for transfer, potentially improving the CLBR in this poor-prognosis population [102–104]. The available evidence suggests a similar euploidy rate, as well as similar LBR and obstetric and perinatal outcomes, in both follicular phase stimulation and luteal phase stimulation cycles in patients undergoing a DuoStim protocol [105]. Recently, the designation of “luteal phase stimulation cycle,” starting five days after oocyte retrieval, has been defied [106]. In fact, due to the premature luteolysis following GnRHa trigger, basal hormone levels are found five days after oocyte retrieval, rendering the terminology “second stimulation in the same ovarian cycle” more appropriate [107]. In this regard, the results of an ongoing randomized controlled trial (NCT03555942) are expected to clarify the clinical impact of performing a luteal-phase stimulation followed by a follicular-phase stimulation cycle in POR patients, combining the advantages of the double stimulation with the possibility of performing a fresh embryo transfer in the same cycle.

Ovarian response category

The freeze-all approach is unarguable in high responders to avoid the risk of OHSS. In these patients, cycle segmentation has shown to be the most effective strategy to prevent this potentially lethal complication [1, 34]. Furthermore, a recent meta-analysis has reported a significantly higher probability of live birth following FET when compared with the fresh ET group in high responders (RR 1.18, 95% CI 1.06–1.31; fixed effects model; 3 RCTs; n = 3398; I² = 0%), as well as a significantly lower miscarriage rate in the FET

group when compared with the fresh ET group (RR 0.69, 95% CI 0.55–0.86; fixed effects model; 2 RCTs; n = 1630; I² = 0%), reinforcing the importance of cycle segmentation in this population [108].

Despite the acknowledged benefit of the freeze-all policy in high responders, the question of whether this approach should be adopted in the general IVF population is still a matter of debate. In fact, two recent meta-analysis have shown similar reproductive outcomes in terms of LBR, miscarriage rate, and CLBR when FET and fresh ET cycles were compared in normo-responders [108, 109]. After the publication of these meta-analysis, three RCTs have been published in normo-responders and have shown a similar healthy baby rate (defined as term, singleton, live birth with appropriate weight for gestation) [110], as well as similar LBR and miscarriage rate [50, 110] following elective FET and fresh ET, while a lower CLBR with elective FET was reported in one of the trials [111].

Finally, albeit of low quality, the available evidence does not seem to support any benefit of elective FET in POR patients in terms of reproductive outcomes [112–115].

In conclusion, a freeze-all policy should not be routinely recommended unless indicated by other clinical factors (e.g. risk of OHSS, PGT, late-follicular phase progesterone elevation, fertility preservation).

Maternal and perinatal risks

The subject of gestational and perinatal complications in FET and fresh ET has been recently reviewed in a Cochrane meta-analysis [53]. A similar prevalence of gestational diabetes, preterm delivery, small-for-gestational-age babies, congenital abnormalities, and perinatal mortality was observed with both approaches. However, an increased risk of hypertensive disorders (OR 2.15, 95% CI 1.42–3.25; 3 RCTs; n = 3940; I² = 29%) and large-for-gestational-age babies (OR 1.96, 95% CI 1.51–2.55; 3 RCTs; n = 3940; I² = 0%) was found following the freeze-all strategy. Despite the fact that pre-eclampsia is a multifactorial disorder, a recent prospective cohort study has demonstrated that women who conceive without a corpus luteum are at increased risk of preeclampsia and severe preeclampsia compared to women who conceive with at least one corpus luteum [116]. Moreover, the authors performed a sub-analysis of FET cycles and reported a higher risk of preeclampsia in patients undergoing hormone replacement therapy cycles when compared to modified natural cycles. These findings have also been corroborated in recent large cohort studies, favouring endometrial preparation regimens with the presence of a corpus luteum [117–119]. The results from these observational trials call for further research to verify whether the adoption of stimulated or natural FET might mitigate the increased risk of hypertensive disorders of pregnancy and increased birth weight associated with FET.

References

- Devroey P, Polyzos NP, Blockeel C. An OHSS-Free Clinic by segmentation of IVF treatment. *Hum Reprod*. 2011;26(10):2593–7.
- Wyns C, Bergh C, Calhaz-Jorge C, De Geyter C, Kupka MS, Motrenko T, et al. ART in Europe, 2016: Results generated from European registries by ESHRE†. *Hum Reprod Open*. 2020;2020(3):1–17.
- Roche K, Racowsky C, Harper J. Utilization of preimplantation genetic testing in the USA. *J Assist Reprod Genet*. 2021;38(5): 1045–53.
- Martinez F, Racca A, Rodríguez I, Polyzos NP. Ovarian stimulation for oocyte donation: A systematic review and meta-analysis. *Hum Reprod Update*. 2021;27(4):673–96.
- Anderson RA, Amant F, Braat D, D'Angelo A, Chuva de Sousa Lopes SM, Demeestere I, et al. ESHRE guideline: Female fertility preservation. *Hum Reprod Open*. 2020;2020(4):1–17.
- Steward RG, Lan L, Shah AA, Yeh JS, Price TM, Goldfarb JM, et al. Oocyte number as a predictor for ovarian hyperstimulation syndrome and live birth: An analysis of 256,381 in vitro fertilization cycles. *Fertil Steril*. 2014;101(4):967–73.
- Sunkara SK, Rittenberg V, Raine-Fenning N, Bhattacharya S, Zamora J, Coomarasamy A. Association between the number of eggs and live birth in IVF treatment: An analysis of 400 135 treatment cycles. *Hum Reprod*. 2011 Jul 1;26(7):1768–74. Available from: <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=21558332&retmode=ref&cmd=prlinks>.
- Maheshwari A, McLernon D, Bhattacharya S. Cumulative live birth rate: Time for a consensus? *Hum Reprod*. 2015;30(12):2703–7. Available from: <https://academic.oup.com/humrep/article-lookup/doi/10.1093/humrep/dev263>.
- Malchau SS, Henningsen AA, Forman J, Loft A, Nyboe Andersen A, Pinborg A. Cumulative live birth rate prognosis based on the number of aspirated oocytes in previous ART cycles. *Hum Reprod*. 2019;34(1):171–80.
- Magnusson Å, Wennerholm UB, Källén K, Petzold M, Thurin-Kjellberg A, Bergh C. The association between the number of oocytes retrieved for IVF, perinatal outcome and obstetric complications. *Hum Reprod*. 2018;33(10):1939–47.
- Polyzos N, Drakopoulos P, Parra J, Pellicer A, Santos-Ribeiro S, Tournaye H, et al. Cumulative live birth rates according to the number of oocytes retrieved after the first ovarian stimulation for in vitro fertilization/intracytoplasmic sperm injection: A multicenter multinational analysis including ~15,000 women. *Fertil Steril*. 2018;110:661–70.e1.
- Drakopoulos P, Blockeel C, Stoop D, Camus M, de Vos M, Tournaye H, et al. Conventional ovarian stimulation and single embryo transfer for IVF/ICSI. How many oocytes do we need to maximize cumulative live birth rates after utilization of all fresh and frozen embryos? *Hum Reprod*. 2016 Jan 2;31(2):370–6. Available from: <https://academic.oup.com/humrep/article-lookup/doi/10.1093/humrep/dev316>.
- Vaughan DA, Leung A, Resetkova N, Ruthazer R, Penzias AS, Sakkas D, et al. How many oocytes are optimal to achieve multiple live births with one stimulation cycle? The one-and-done approach. *Fertil Steril*. 2017 Feb 1;107(2):397–404.e3. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0015028216629606>.
- Cobo A, Garrido N, Pellicer A, Remohí J. Six years 'experience in ovum donation using vitrified oocytes : Report of cumulative outcomes, impact of storage time, and development of a predictive model for oocyte survival rate. *Fertil Steril*. 2015;104(6):1426–34.e1–8.
- Al-Inany HG, Youssef MA, Ayeleke RO, Brown J, Lam WS, Broekmans FJ. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev*. 2016;4:CD001750. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/27126581>.
- Toftager M, Bogstad J, Bryndorf T, Løssl K, Roskær J, Holland T, et al. Risk of severe ovarian hyperstimulation syndrome in GnRH antagonist versus GnRH agonist protocol: RCT including 1050 first IVF/ICSI cycles. *Hum Reprod*. 2016;31(6):1253–64.
- Huirne JA, Homburg R, Lambalk CB. Are GnRH antagonists comparable to agonists for use in IVF? *Hum Reprod*. 2007;22(11): 2805–13.
- Toftager M, Bogstad J, Løssl K, Prætorius L, Zedeler A, Bryndorf T, et al. Cumulative live birth rates after one ART cycle including all subsequent frozen-thaw cycles in 1050 women: Secondary outcome of an RCT comparing GnRH-antagonist and GnRH-agonist protocols. *Hum Reprod*. 2017;32(3):556–67. Available from: <https://academic.oup.com/humrep/article-lookup/doi/10.1093/humrep/dew358>.

19. Kuang Y, Chen Q, Fu Y, Wang Y, Hong Q, Lyu Q, et al. Medroxyprogesterone acetate is an effective oral alternative for preventing premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation for in vitro fertilization. *Fertil Steril.* 2015;104(1):62–70.e3. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0015028215002277>.
20. Hamdi K, Farzadi L, Ghasemzadeh A, Navali N, Atashkhoei S, Pia H, et al. Comparison of medroxyprogesterone acetate with cetrodote for prevention of premature luteinizing hormone surges in women undergoing in vitro fertilization. *Int J Women's Heal Reprod Sci.* 2018;6(2):187–91. DOI: [10.15296/ijwhr.2018.31](https://doi.org/10.15296/ijwhr.2018.31).
21. Beguería R, García D, Vassena R, Rodríguez A. Medroxyprogesterone acetate versus ganirelix in oocyte donation: A randomized controlled trial. *Hum Reprod.* 2019;34(5):872–80.
22. Yıldız S, Turkgeldi E, Angun B, Eraslan A, Urman B, Ata B. Comparison of a novel flexible progestin primed ovarian stimulation protocol and the flexible gonadotropin-releasing hormone antagonist protocol for assisted reproductive technology. *Fertil Steril.* 2019;112(4):677–83. DOI: [10.1016/j.fertnstert.2019.06.009](https://doi.org/10.1016/j.fertnstert.2019.06.009).
23. Giles J, Alama P, Gamiz P, Vidal C, Badia P, Pellicer A, et al. Medroxyprogesterone acetate is a useful alternative to a gonadotropin-releasing hormone antagonist in oocyte donation: A randomized, controlled trial. *Fertil Steril.* 2021;116(2):404–12. DOI: [10.1016/j.fertnstert.2021.02.036](https://doi.org/10.1016/j.fertnstert.2021.02.036).
24. Yu S, Long H, Ning Chang Y-, Liu H, Gao Y, Zhu H J, et al. New application of dydrogesterone as a part of a progestin-primed ovarian stimulation protocol for IVF: A randomized controlled trial including 516 first IVF/ICSI cycles. *Hum Reprod.* 2018;33(2):229–37.
25. Zhu X, Zhang X, Fu Y. Utrigestan as an effective Oral alternative for preventing premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation for in vitro fertilization. *Medicine (Baltimore).* 2015;94(21):1–8.
26. Zhu X, Ye H, Fu Y. The utrigestan and hMG protocol in patients with polycystic ovarian syndrome undergoing controlled ovarian hyperstimulation during IVF/ICSI treatments. *Medicine (Baltimore).* 2016;95–28.
27. Martínez F, Rodriguez-Purata J, Beatriz Rodríguez D, Clua E, Rodriguez I, Coroleu B. Desogestrel versus antagonist injections for LH suppression in oocyte donation cycles: A crossover study. *Gynecol Endocrinol.* 2019;35(10):878–3. DOI: [10.1080/09513590.2019.1604661](https://doi.org/10.1080/09513590.2019.1604661).
28. Martínez F, Rodriguez-Purata J, Clua E, Garcia S, Coroleu B, Polyzos N. Ovarian response in oocyte donation cycles under LH suppression with GnRH antagonist or desogestrel progestin: Retrospective and comparative study. *Gynecol Endocrinol.* 2019;35(10):884–9. DOI: [10.1080/09513590.2019.1604662](https://doi.org/10.1080/09513590.2019.1604662).
29. Massin N. New stimulation regimens: Endogenous and exogenous progesterone use to block the LH surge during ovarian stimulation for IVF. *Hum Reprod Update.* 2017;23(2):211–20. Available from: <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=28062551&retmode=ref&cmd=prlinks>.
30. Mizrachi Y, Horowitz E, Farhi J, Raziel A, Weissman A. Ovarian stimulation for freeze-all IVF cycles: A systematic review. *Hum Reprod Update.* 2020;26(1):119–36.
31. Fauzer BC, de Jong D, Olivennes F, Wrambsy H, Tay C, Itskovitz-Eldor J, et al. Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for in vitro fertilization. *J Clin Endocrinol Metab.* 2002;87(2):709–15. Available from: <https://academic.oup.com/jcem/article-lookup/doi/10.1210/jcem.87.2.8197>.
32. Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, Kolibianakis E, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI. *Hum Reprod Open.* 2020;2020(2):1–13.
33. Youssef M, Van der Veen F, Al-Inany H, Mochtar M, Griesinger G, Mohesen N, et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology (review). *Cochrane Database Syst Rev.* 2014;10:CD008046.
34. Griesinger G, Schultz L, Bauer T, Broessner A, Frambach T, Kissler S. Ovarian hyperstimulation syndrome prevention by gonadotropin-releasing hormone agonist triggering of final oocyte maturation in a gonadotropin-releasing hormone antagonist protocol in combination with a “freeze-all” strategy: A prospective multicentric. *Fertil Steril.* 2011;95(6):2029–2033.e1. DOI: [10.1016/j.fertnstert.2011.01.163](https://doi.org/10.1016/j.fertnstert.2011.01.163).
35. Humaidan P, Bredkjær HE, Bungum L, Bungum M, Grøndahl ML, Westergaard L, et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomized study. *Hum Reprod.* 2005;20(5):1213–20.
36. Lan KC, Chen YC, Lin YC, Tsai YR. Gonadotrophin-releasing hormone agonist triggering may improve central oocyte granularity and embryo quality. *Zygote.* 2020;28(4):337–43.
37. Fatemi HM, Popovic-Todorovic B, Humaidan P, Kol S, Bunker M, Devroey P, et al. Severe ovarian hyperstimulation syndrome after gonadotropin-releasing hormone (GnRH) agonist trigger and “freeze-all” approach in GnRH antagonist protocol. *Fertil Steril.* 2014;101(4):1008–11. DOI: [10.1016/j.fertnstert.2014.01.019](https://doi.org/10.1016/j.fertnstert.2014.01.019).
38. Santos-Ribeiro S, Polyzos N, Stouffs K, De Vos M, Seneca S, Tournaye H, et al. Ovarian hyperstimulation syndrome after gonadotropin-releasing hormone agonist triggering and “freeze-all”: In-depth analysis of genetic predisposition. *J Assist Reprod Genet.* 2015;32(7):1063–8.
39. Orvieto R. Triggering final follicular maturation- hCG, GnRH-agonist or both, when and to whom? *J Ovarian Res.* 2015;8(1):4–9. DOI: [10.1186/s13048-015-0187-6](https://doi.org/10.1186/s13048-015-0187-6).
40. Griffin D, Feinn R, Engmann L, Nulsen J, Budinetz T, Benadiva C. Dual trigger with gonadotropin-releasing hormone agonist and standard dose human chorionic gonadotropin to improve oocyte maturity rates. *Fertil Steril.* 2014;102(2):405–9. Available from: [https://linkinghub.elsevier.com/retrieve/pii/S0015028214003884](http://linkinghub.elsevier.com/retrieve/pii/S0015028214003884).
41. Lin MH, Shao-Ying Wu F, Kuo-Kuang Lee R, Li SH, Lin SY, Hwu YM. Dual trigger with combination of gonadotropin-releasing hormone agonist and human chorionic gonadotropin significantly improves the live-birth rate for normal responders in GnRH-antagonist cycles. *Fertil Steril.* 2013;100(5):1296–302. DOI: [10.1016/j.fertnstert.2013.07.1976](https://doi.org/10.1016/j.fertnstert.2013.07.1976).
42. Ding N, Liu X, Jian Q, Liang Z, Wang F. Dual trigger of final oocyte maturation with a combination of GnRH agonist and hCG versus a hCG alone trigger in GnRH antagonist cycle for in vitro fertilization: A systematic review and meta-analysis. *Eur J Obstet Gynecol Reprod Biol.* 2017;218:92–8. DOI: [10.1016/j.ejogrb.2017.09.004](https://doi.org/10.1016/j.ejogrb.2017.09.004).
43. Haas J, Bassil R, Samara N, Zilberberg E, Mehta C, Orvieto R, et al. GnRH agonist and hCG (dual trigger) versus hCG trigger for final follicular maturation: A double-blinded, randomized controlled study. *Hum Reprod.* 2020;35(7):1648–54.
44. Zhu H, Zhao C, Pan Y, Zhou H, Xu JX W, et al. Dual trigger for final follicular maturation improves cumulative live-birth rate in ovarian stimulation for freeze-all in vitro Fertilization/Intracytoplasmic sperm injection cycles. *Front Endocrinol (Lausanne).* 2021;12(July):1–8.
45. Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Hum Reprod Update.* 2012;18(5):536–54.
46. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: Systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update.* 2017;23(2):139–55.
47. Cobo A, Diaz C. Clinical application of oocyte vitrification: A systematic review and meta-analysis of randomized controlled trials. *Fertil Steril.* 2011;96(2):277–85.

48. Wei D, Liu JY, Sun Y, Shi Y, Zhang B, Liu JQ, et al. Frozen versus fresh single blastocyst transfer in ovulatory women: A multicentre, randomised controlled trial. *Lancet.* 2019;393(10178):1310–8. DOI: [10.1016/S0140-6736\(18\)32843-5](https://doi.org/10.1016/S0140-6736(18)32843-5).
49. Shi Y, Sun Y, Hao C, Zhang H, Wei D, Zhang Y, et al. Transfer of fresh versus frozen embryos in ovulatory women. *N Engl J Med.* 2018 Jan 11;378(2):126–36. Available from: <http://www.nejm.org/doi/10.1056/NEJMoa1705334>.
50. Stormlund S, Sopa N, Zedeler A, Bogstad J, Prätorius L, Nielsen HS, et al. Freeze-all versus fresh blastocyst transfer strategy during in vitro fertilisation in women with regular menstrual cycles: Multicentre randomised controlled trial. *BMJ.* 2020;370:m2519.
51. Saket Z, Källén K, Lundin K, Magnusson Å, Bergh C. Cumulative live birth rate after IVF: Trend over time and the impact of blastocyst culture and vitrification. *Hum Reprod Open.* 2021;2021(3):1–9.
52. Mourad S, Brown J, Farquhar C. Interventions for the prevention of OHSS in ART cycles: An overview of Cochrane reviews. *Cochrane Database Syst Rev.* 2017; 1 (1):CD012103
53. Zaai T, Zagers M, Mol F, Goddijn M, van Wely M, Mastenbroek S. Fresh versus frozen embryo transfers in assisted reproduction. *Cochrane Database Syst Rev.* 2021; 2(2):CD011184
54. Gluovsky D, Farquhar C, Retamar Q, Sedo A, Blake CR D. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev.* 2016; 5(5): CD002118
55. Holden EC, Kashani BN, Morelli SS, Alderson D, Jindal SK, Ohman-Strickland PA, et al. Improved outcomes after blastocyst-stage frozen-thawed embryo transfers compared with cleavage stage: A society for assisted reproductive technologies clinical outcomes reporting system study. *Fertil Steril.* 2018;110(1):89–94.e2. DOI: [10.1016/j.fertnstert.2018.03.033](https://doi.org/10.1016/j.fertnstert.2018.03.033).
56. Fransasi JM, Forman EJ, Patounakis G, Hong KH, Werner MD, Upham KM, et al. Investigating the impact of the timing of blastulation on implantation: Management of embryo-endometrial synchrony improves outcomes. *Hum Reprod Open.* 2018;2018(4):1–6.
57. ESHRE Special Interest Group of Embryology; Alpha Scientists in Reproductive Medicine. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Hum Reprod.* 2011;26(6):1270–83.
58. Committee P, Society A, Committee P, Reproductive A. Blastocyst culture and transfer in clinically assisted reproduction: A committee opinion. *Fertil Steril.* 2018;110(7):1246–52. DOI: [10.1016/j.fertnstert.2018.09.011](https://doi.org/10.1016/j.fertnstert.2018.09.011).
59. Hernandez-Nieto C, Lee JA, Slifkin R, Sandler B, Copperman AB, Flisser E. What is the reproductive potential of day 7 euploid embryos. *Hum Reprod.* 2019;34(9):1697–706.
60. Tiegs AW, Sun L, Patounakis G, Scott RT. Worth the wait day 7 blastocysts have lower euploidy rates but similar sustained implantation rates as day 5 and day 6 blastocysts. *Hum Reprod.* 2019;34(9):1632–9.
61. Du T, Wang Y, Fan Y, Zhang S, Yan Z, Yu W, et al. Fertility and neonatal outcomes of embryos achieving blastulation on day 7: Are they of clinical value? *Hum Reprod.* 2018;33(6):1038–51.
62. Huang J, Yang X, Wu J, Kuang Y, Wang Y. Impact of day 7 blastocyst transfer on obstetric and perinatal outcome of singletons born after vitrified-warmed embryo transfer. *Front Physiol.* 2020;11(February):1–10.
63. Kovalevsky G, Carney SM, Morrison LS, Boylan CF, Neithardt AB, Feinberg RF. Should embryos developing to blastocysts on day 7 be cryopreserved and transferred: An analysis of pregnancy and implantation rates. *Fertil Steril.* 2013;100(4):1008–12. DOI: [10.1016/j.fertnstert.2013.06.021](https://doi.org/10.1016/j.fertnstert.2013.06.021).
64. Hammond ER, Cree LM, Morbeck DE. Should extended blastocyst culture include day 7? *Hum Reprod.* 2018;33(6):991–7.
65. Centers for Disease Control and Prevention. 2019 Assisted Reproductive Technology Fertility Clinic and National Summary Report. US Dept Heal Hum Serv. 2021.
66. Munne S, Dailey T, Sultan K, Grifo J, Cohen J. Diagnosing and preventing inherited disease: The use of first polar bodies for preimplantation diagnosis of aneuploidy. *Hum Reprod.* 1995;10(4): 1014–20.
67. Gianaroli L, Magli MC, Ferraretti AP, Munné S. Preimplantation diagnosis for aneuploidies in patients undergoing in vitro fertilization with a poor prognosis: Identification of the categories for which it should be proposed. *Fertil Steril.* 1999;72(5):837–44.
68. Scott KL, Hong KH, Scott RT. Selecting the optimal time to perform biopsy for preimplantation genetic testing. *Fertil Steril.* 2013;100(3):608–14. DOI: [10.1016/j.fertnstert.2013.07.004](https://doi.org/10.1016/j.fertnstert.2013.07.004).
69. Scott RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: A randomized and paired clinical trial. *Fertil Steril.* 2013;100(3):624–30. DOI: [10.1016/j.fertnstert.2013.04.039](https://doi.org/10.1016/j.fertnstert.2013.04.039).
70. Coates A, Kung A, Mounts E, Hesla J, Bankowski B, Barbieri E, et al. Optimal euploid embryo transfer strategy, fresh versus frozen, after preimplantation genetic screening with next generation sequencing: A randomized controlled trial. *Fertil Steril.* 2017;107(3):723–730.e3.
71. Venetis CA, Kolibianakis EM, Bosdou JK, Tarlatzis BC. Progesterone elevation and probability of pregnancy after IVF: A systematic review and meta-analysis of over 60 000 cycles. *Hum Reprod Update.* 2013;19(5):433–57. Available from: <https://academic.oup.com/humupd/article-lookup/doi/10.1093/humupd/dmt014>.
72. Venetis CA, Kolibianakis EM, Bosdou JK, Tarlatzis BC. Estimating the net effect of progesterone elevation on the day of hCG on live birth rates after IVF: A cohort analysis of 3296 IVF cycles. *Hum Reprod.* 2015 Feb 12;30(3):684–91. Available from: <https://academic.oup.com/humrep/article-lookup/doi/10.1093/humrep/deu362>.
73. Bosch E, Labarta E, Crespo J, Simón C, Remohí J, Jenkins J, et al. Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for in vitro fertilization: Analysis of over 4000 cycles. *Hum Reprod.* 2010;25(8): 2092–100.
74. Santos-Ribeiro S, Polyzos NP, Haentjens P, Smitz J, Camus M, Tournaye H, et al. Live birth rates after IVF are reduced by both low and high progesterone levels on the day of human chorionic gonadotrophin administration. *Hum Reprod.* 2014 Aug 1;29(8):1698–705. Available from: <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=24939956&retmode=ref&cmd=prlinks>.
75. Fleming R, Jenkins J. The source and implications of progesterone rise during the follicular phase of assisted reproduction cycles. *Reprod Biomed Online.* 2010;21:446–9.
76. Oktem O, Akin N, Bildik G, Yakin K, Alper E, Balaban B, et al. FSH stimulation promotes progesterone synthesis and output from human granulosa cells without luteinization. *Hum Reprod.* 2017; 32:643–52.
77. Healy MW, Yamasaki M, Patounakis G, Richter KS, Devine K, DeCherney AH, et al. The slow growing embryo and premature progesterone elevation: Compounding factors for embryo-endometrial asynchrony. *Hum Reprod.* 2016;32:362–7.
78. Martinez F, Rodriguez I, Devesa M, Buxaderas R, Gómez MJ, Coroleu B. Should progesterone on the human chorionic gonadotropin day still be measured? *Fertil Steril.* 2015;105: 86–92.
79. Xu B, Li Z, Zhang H, Jin L, Li Y, Ai J, et al. Serum progesterone level effects on the outcome of in vitro fertilization in patients with different ovarian response: An analysis of more than 10,000 cycles. *Fertil Steril.* 2012;97:1321–7.e4.
80. Horcajadas J, Riesewijk A, Polman J, Van Os R, Pellicer A, Mosselman S, et al. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. *Mol Hum Reprod.* 2005;11:195–205.

81. Labarta E, Martínez-Conejero JA, Alamá P, Horcajadas JA, Pellicer A, Simón C, et al. Endometrial receptivity is affected in women with high circulating progesterone levels at the end of the follicular phase: A functional genomics analysis. *Hum Reprod.* 2011;26:1813–25.
82. Xiong Y, Wang J, Liu L, Chen X, Xu H, Li TC, et al. Effects of high progesterone level on the day of human chorionic gonadotrophin administration in in vitro fertilization cycles on epigenetic modification of endometrium in the peri-implantation period. *Fertil Steril.* 2017;108:269–276.e1.
83. Van Vaerenbergh I, Fatemi HM, In't Veld P, Blockeel C, Van Lommel L, In't Veld P, et al. Progesterone rise on HCG day in GnRH antagonist/rFSH stimulated cycles affects endometrial gene expression. *Reprod Biomed Online.* 2011;22:263–71.
84. Baldini D, Savoia M, Sciancalepore A, Malvasi A, Vizziello D, Beck R, et al. High progesterone levels on the day of HCG administration do not affect the embryo quality and the reproductive outcomes of frozen embryo transfers. *Clin Ter.* 2018;169:e91–5.
85. Neves AR, Santos-Ribeiro S, García-Martínez S, Devesa M, Soares SR, García-Velasco JA, et al. The effect of late-follicular phase progesterone elevation on embryo ploidy and cumulative live birth rates. *Reprod Biomed Online.* 2021;43(6):1063–9. DOI: [10.1016/j.rbmo.2021.07.019](https://doi.org/10.1016/j.rbmo.2021.07.019).
86. Healy MW, Patounakis G, Connell MT, Devine K, DeCherney AH, Levy MJ, et al. Does a frozen embryo transfer ameliorate the effect of elevated progesterone seen in fresh transfer cycles? *Fertil Steril.* 2016 Jan 1;105(1):93–99.e1. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0015028215019329>.
87. Racca A, Vanni V, Somigliana E, Reschini M, Viganò P, Santos-Ribeiro S, et al. Is a freeze-all policy the optimal solution to circumvent the effect of late follicular elevated progesterone? A multicentric matched-control retrospective study analysing cumulative live birth rate in 942 non-elective freeze-all cycles. *Hum Reprod.* 2021;36(9):2463–72.
88. Hernandez-Nieto C, Lee JA, Alkon-Meadows T, Luna-Rojas M, Mukherjee T, Copperman AB, et al. Late follicular phase progesterone elevation during ovarian stimulation is not associated with decreased implantation of chromosomally screened embryos in thaw cycles. *Hum Reprod.* 2020;35(8):1889–99.
89. Huang B, Ren X, Wu L, Zhu L, Xu B, Li Y, et al. Elevated progesterone levels on the day of oocyte maturation May affect top quality embryo IVF cycles. Sun q-y, editor. *PLoS One.* 2016;11(1):e0145895. <http://dx.plos.org/10.1371/journal.pone.0145895>.
90. Racca A, Santos-Ribeiro S, De Munck N, Mackens S, Drakopoulos P, Camus M, et al. Impact of late-follicular phase elevated serum progesterone on cumulative live birth rates: Is there a deleterious effect on embryo quality? *Hum Reprod.* 2018 May 1;33(5):860–8. Available from: <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=29481670&retmode=ref&cmd=prlinks>.
91. Vanni VS, Somigliana E, Reschini M, Pagliardini L, Marotta E, Faulisi S, et al. Top quality blastocyst formation rates in relation to progesterone levels on the day of oocyte maturation in GnRH antagonist IVF/ICSI cycles. Kim s, editor. *PLoS One.* 2017 May 17;12(5):e0176482. <http://dx.plos.org/10.1371/journal.pone.0176482>.
92. Maman E, Meirow D, Brengauz M, Raanani H, Dor J, Hourvitz A. Luteal phase oocyte retrieval and in vitro maturation is an optional procedure for urgent fertility preservation. *Fertil Steril.* 2011;95(1):64–7. DOI: [10.1016/j.fertnstert.2010.06.064](https://doi.org/10.1016/j.fertnstert.2010.06.064).
93. Baerwald AR, Adams GP, Pierson RA. A new model for ovarian follicular development during the human menstrual cycle. *Fertil Steril.* 2003;80(1):116–22.
94. Nayak SR, Wakim AN. Random-start gonadotropin-releasing hormone (GnRH) antagonist-treated cycles with GnRH agonist trigger for fertility preservation. *Fertil Steril.* 2011;96(1):e51–4. DOI: [10.1016/j.fertnstert.2011.04.079](https://doi.org/10.1016/j.fertnstert.2011.04.079).
95. Buendgen N, Schultze-Mosgau A, Cordes T, Diedrich K, Griesinger G. Initiation of ovarian stimulation independent of the menstrual cycle: A case-control study. *Arch Gynecol Obs.* 2013;288(4):901–4.
96. Qin N, Chen Q, Hong Q, Cai R, Gao H, Wang Y, et al. Flexibility in starting ovarian stimulation at different phases of the menstrual cycle for treatment of infertile women with the use of in vitro fertilization or intracytoplasmic sperm injection. *Fertil Steril.* 2016;106(2):334–341.e1.
97. Zhu X, Fu Y. Evaluation of ovarian stimulation initiated from the late follicular phase using human menopausal gonadotropin alone in normal-ovulatory women for treatment of infertility: A retrospective cohort study. *Front Endocrinol (Lausanne).* 2019;10(JULY):1–9.
98. Boots CE, Meister M, Cooper AR, Hardi A, Jungheim ES. Ovarian stimulation in the luteal phase: Systematic review and meta-analysis. *J Assist Reprod Genet.* 2016;33(8):971–80. DOI: [10.1007/s10815-016-0721-5](https://doi.org/10.1007/s10815-016-0721-5).
99. Martínez F, Clua E, Devesa M, Rodríguez I, Arroyo G, González C, et al. Comparison of starting ovarian stimulation on day 2 versus day 15 of the menstrual cycle in the same oocyte donor and pregnancy rates among the corresponding recipients of vitrified oocytes. *Fertil Steril.* 2014;102(5):1307–11.
100. Chen H, Wang Y, Lyu Q, Ai A, Fu Y, Tian H, et al. Comparison of live-birth defects after luteal-phase ovarian stimulation vs. conventional ovarian stimulation for in vitro fertilization and vitrified embryo transfer cycles. *Fertil Steril.* 2015;103(5):1194–1201.e2.
101. Wang N, Wang Y, Chen Q, Dong J, Tian H, Fu Y, et al. Luteal-phase ovarian stimulation vs conventional ovarian stimulation in patients with normal ovarian reserve treated for IVF: A large retrospective cohort study. *Clin Endocrinol (Oxf).* 2016;84(5):720–8.
102. Kuang Y, Chen Q, Hong Q, Lyu Q, Ai A, Fu Y, et al. Double stimulations during the follicular and luteal phases of poor responders in IVF/ICSI programmes (Shanghai protocol). *Reprod Biomed Online.* 2014;29(6):684–91.
103. Ubaldi FM, Capalbo A, Vaiarelli A, Cimadomo D, Colamaria S, Alviggi C, et al. Follicular versus luteal phase ovarian stimulation during the same menstrual cycle (DuoStim) in a reduced ovarian reserve population results in a similar euploid blastocyst formation rate: New insight in ovarian reserve exploitation. *Fertil Steril.* 2016;105(6):1488–95.e1. [https://linkinghub.elsevier.com/retrieve/pii/S0015028216300024](http://linkinghub.elsevier.com/retrieve/pii/S0015028216300024).
104. Vaiarelli A, Cimadomo D, Conforti A, Schimberni M, Giuliani M, D'Alessandro P, et al. Luteal phase after conventional stimulation in the same ovarian cycle might improve the management of poor responder patients fulfilling the Bologna criteria: A case series. *Fertil Steril.* 2020;113(1):121–30. DOI: [10.1016/j.fertnstert.2019.09.012](https://doi.org/10.1016/j.fertnstert.2019.09.012).
105. Vaiarelli A, Cimadomo D, Alviggi E, Sansone A, Trabucco E, Dusi L, et al. The euploid blastocysts obtained after luteal phase stimulation show the same clinical, obstetric and perinatal outcomes as follicular phase stimulation-derived ones: A multicenter study. *Hum Reprod.* 2020;35(11):2598–608.
106. Racca A, Polyzos NP. DuoStim: Are we really comparing follicular phase with luteal phase stimulations? *Hum Reprod.* 2021;36(6):1722–3.
107. Vaiarelli A, Cimadomo D, Rienzi L, Ubaldi F. Reply: 'Second stimulation in the same ovarian cycle', probably a terminology more appropriate than 'luteal phase stimulation' in the DuoStim protocol. *Hum Reprod.* 2021;36(6):1723–24.
108. Bosdou JK, Venetis CA, Tarlatzis BC, Grimbizis GF, Kolibianakis EM. Higher probability of live-birth in high, but not normal, responders after first frozen-embryo transfer in a freeze-only cycle strategy compared to fresh-embryo transfer: A meta-analysis. *Hum Reprod.* 2019;34(3):491–505.
109. Roque M, Haahr T, Geber S, Esteves SC, Humaidan P. Fresh versus elective frozen embryo transfer in IVF/ICSI cycles: A systematic review and meta-analysis of reproductive outcomes. *Hum Reprod Update.* 2019;25(1):2–14.
110. Maheshwari A, Bell JL, Bhide P, Brison D, Child T, Chong HY, et al. Elective freezing of embryos versus fresh embryo transfer in IVF: A multicentre randomized controlled trial in the UK (E-freeze). *Hum Reprod.* 2022;37(3):476–87.

111. Wong KM, Van Wely M, Verhoeve HR, Kaaijk EM, Mol F, Van Der Veen F, et al. Transfer of fresh or frozen embryos: A randomised controlled trial. *Hum Reprod.* 2021;36(4):998–1006.
112. Roque M, Valle M, Sampaio M, Geber S. Does freeze-all policy affect IVF outcome in poor ovarian responders? *Ultrasound Obstet Gynecol.* 2018;52:530–4.
113. Liu C, Li Y, Jiang H, Liu Y, Song X. The clinical outcomes of fresh versus frozen embryos transfer in women ≥ 40 years with poor ovarian response. *Obstet Gynecol Sci.* 2021;64(3):284–92.
114. Çelik S, Turgut NE, Yağmur E, Boynukalın K, Çelik DC, Fındıklı N, et al. Bologna kriterlerine uyan zayıf cevaplı kadınlarda taze ve elektif dondurulmuş/çözülmüş embriyo transferlerinin gebelik sonuçlarına etkisi. *Turk Jinekoloji ve Obstet Dern Derg.* 2015;12(3):132–8.
115. Berkkanoglu M, Coetzee K, Bulut H, Ozgur K. Optimal embryo transfer strategy in poor response may include freeze-all. *J Assist Reprod Genet.* 2017;34(1):79–87. DOI: [10.1007/s10815-016-0825-y](https://doi.org/10.1007/s10815-016-0825-y).
116. Von Versen-Hoynck F, Schaub AM, Chi YY, Chiu KH, Liu J, Lingis M, et al. Increased preeclampsia risk and reduced aortic compliance with in vitro fertilization cycles in the absence of a corpus luteum. *Hypertension.* 2019;73(3):640–9.
117. Zhang J, Wei M, Bian X, Wu L, Zhang S, Mao X, et al. Letrozole-induced frozen embryo transfer cycles are associated with a lower risk of hypertensive disorders of pregnancy among women with polycystic ovary syndrome. *Am J Obstet Gynecol.* 2021;225(1):59. e1–e9. DOI: [10.1016/j.ajog.2021.01.024](https://doi.org/10.1016/j.ajog.2021.01.024).
118. Ginström Ernstad E, Wennerholm UB, Khatibi A, Petzold M, Bergh C. Neonatal and maternal outcome after frozen embryo transfer: Increased risks in programmed cycles. *Am J Obstet Gynecol.* 2019;221(2):126.e1–e18. DOI: [10.1016/j.ajog.2019.03.010](https://doi.org/10.1016/j.ajog.2019.03.010).
119. Afferhoj LL, Spangmose AL, Aaris Henningsen AK, Clausen TD, Ziebe S, Jensen RB, et al. Adverse obstetric and perinatal outcomes in 1,136 singleton pregnancies conceived after programmed frozen embryo transfer (FET) compared with natural cycle FET. *Fertil Steril.* 2021;115(4):947–56. DOI: [10.1016/j.fertnstert.2020.10.039](https://doi.org/10.1016/j.fertnstert.2020.10.039).

CONTROLLED OVARIAN STIMULATION FOR FREEZE-ALL CYCLES

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Introduction

Advances in freezing techniques have led to an increase in the prevalence of “freeze-all” cycles, in which all cohort of eggs/embryos is being frozen, and there is no transfer of fresh embryos. In the United States, for example, 37% of all controlled ovarian stimulation (COS) cycles performed in 2019 were banking cycles, where eggs or embryos were frozen and stored for potential future use [1]. This practice is also termed “elective frozen embryo transfer” or “cycle segmentation.”

Several clinical conditions may indicate a freeze-all approach. Some evidence has suggested that elective freezing of all embryos and subsequent frozen-thawed embryo transfer (FET) might be beneficial in patients with a high response to gonadotropins (high responders), and result in a higher live birth rate (LBR) in this group [2, 3]. On the contrary, a practice of routine elective freeze-all probably does not increase the LBR in normal responders [4, 5] or poor responders [6].

The use of pre-implantation genetic testing for aneuploidy (PGT-A) is also regaining widespread popularity and, like pre-implantation genetic testing for monogenic disorders (PGT-M), often requires elective embryo freezing until test results are available. Fertility preservation, for either medical or “social” indications, is another frequent indication for cycle segmentation. Oocyte donation cycles for either fresh embryo transfer or embryo banking, and COS for surrogacy, are other examples of cycles in which the patient who underwent COS does not have a replacement of fresh embryos.

Freeze-all approach has also been suggested to improve the reproductive outcome in cases of recurrent implantation failure (RIF) [7]. It is mandatory in non-conventional ovarian stimulation protocols, such as “random start” and “double stimulation.” Finally, there are certain clinical circumstances under which fresh *in vitro* fertilization (IVF) cycles might be converted to segmented cycles, such as in case of late follicular phase progesterone elevation, high risk for severe ovarian hyperstimulation syndrome (OHSS), inadequate endometrial development, and other medical and paramedical considerations. Common indications for cycle segmentation are summarized in Table 46.1.

In this chapter, we aimed to describe the best available evidence for all components of COS in freeze-all cycles; the optimal size of the follicular cohort, the method of pituitary suppression, the type and dose of gonadotropins, monitoring considerations, criteria for ovulation triggering, and type of the ovulatory trigger, which may all be different compared to fresh cycles. Studies reporting the outcome of elective freeze-all cycles were predominantly used. In addition, studies on oocyte donation (OD) cycles were also considered a good model, since the COS and embryo transfer phases are completely separated. This chapter is based on a previous systematic review we published in 2020 [8], and on more recent publications.

Optimal number of oocytes retrieved in freeze-all cycle

The question of how many oocytes we should aim to retrieve in an IVF cycle was examined in several studies. However, most of these studies only examined the outcome of fresh IVF cycles. In fresh cycles, the LBR reaches a plateau [9] or even declines [10] once more than 15–20 oocytes are retrieved. When planning for a fresh transfer, a delicate balance exists between efficacy and safety. While an increased number of oocytes is associated with an increase in LBR, it is also associated with an increased risk of OHSS [9, 11]. Therefore, 10–15 oocytes have become a widely accepted target in fresh IVF cycles.

One of the advantages of freeze-all cycles is that the risk of severe OHSS can be virtually eliminated. In such case, it may be possible to increase the target number of oocytes to be retrieved. Freeze-all cycles also require a shift in the way a cycle’s outcome is reported [12, 13]. Rather than reporting the LBR per started cycle, the cumulative live birth rate (CLBR) may be a more appropriate outcome measure [14].

Three retrospective cohort studies have reported the CLBR following freeze-all or oocyte donation (OD) cycles. Zhu et al. [15] reported the CLBR in 20,687 women undergoing their first IVF cycle using a freeze-all strategy. The CLBR was positively correlated with the number of oocytes retrieved and increased as the number of oocytes retrieved increased up to 25. In contrast, Ozgur et al. [16] reported the CLBR of one complete freeze-all cycle in 1582 patients in a general infertile population during 18 months following oocyte retrieval. While the CLBR was 55.0%, the number of retrieved oocytes was not independently predictive of CLBR. Cobo et al. [17] have reported their experience in ovum donors using vitrified oocytes. The CLBR increased progressively as the number of oocytes retrieved increased, reaching 97.3% with the retrieval of 43 oocytes.

Several large retrospective cohort studies have reported the CLBR of fresh and FET cycles. The CLBR significantly increased with the number of oocytes retrieved, in both GnRH-agonist [18] and GnRH-antagonist [19, 20] cycles. Magnusson et al. [11] have reported the results of a large national Swedish cohort of 39,387 women. The CLBR increased up to 20 oocytes retrieved, reaching 45.8%. In another large retrospective cohort study, the CLBR steadily increased with the number of oocytes, reaching 70% when ≥ 25 oocytes were retrieved [20]. Interestingly, no plateau in cumulative live birth rates was observed, but a moderate increase of 5.1% on average was detected beyond 27 oocytes. Vaughan et al. [21] evaluated how many oocytes should be retrieved to achieve more than one live birth with one stimulation cycle. It was found that as the number of oocytes retrieved increased, the chance of at least two live births increased, with odds ratio of 1.08 (8% increase in live birth per additional oocyte).

TABLE 46.1 Common Indications for Cycle Segmentation (“Freeze-All”)

- Elective routine cycle segmentation in hyper-responders
- PGT-A or PGT-M
- Fertility preservation for medical or social indications
- Oocyte donation and surrogacy
- RIF
- “Random-start” or “double-stimulation” cycles
- Late follicular phase progesterone elevation
- Risk of OHSS
- Inadequate endometrial development

Abbreviations: PGT-A, pre-implantation genetic testing for aneuploidy; PGT-M, pre-implantation genetic testing for monogenic disorders; RIF, recurrent implantation failure; OHSS, ovarian hyperstimulation syndrome.

To the best of our knowledge, there are no RCTs examining the question of what should be the target number of oocytes retrieved. Large retrospective studies on oocyte numbers and the likelihood of live birth are subjected to bias, because women with high ovarian response may inherently have a better prognosis. Thus, the evidence should be carefully reviewed.

Possible drawbacks of a large cohort of follicles

The development of a very large cohort of follicles may have some drawbacks. Patients are often in considerable pain and discomfort following the retrieval of a large cohort of follicles. The extremely high estradiol (E2) levels are associated with a slight increase in the rare incidence of thromboembolic events [11]. In addition, there may be an increased risk of bleeding after excessive punctures to remove a large number of oocytes (>30) [22], and ovarian enlargement after retrieval may predispose patients to adnexal torsion. Finally, there is a potential for a disaster in case a patient mistakenly receives hCG instead of GnRH agonist for ovulation triggering.

Summary

The target number of oocytes should be individualized according to patient’s age, ovarian reserve, and clinical circumstances. Although there is strong evidence to suggest that the CLBR increases with the number of retrieved oocytes, there are concerns regarding the patient’s safety and recovery after excessive stimulation. Therefore, for most patients, the best estimate would be to aim for a retrieval of between 15 and 20 oocytes in freeze-all cycles, which represents a good balance between safety and efficacy.

Pre-treatment interventions: Practical and medical considerations

A variety of hormone pre-treatment regimens have been described in order to improve the synchronization of the follicular cohort, to prevent early emergence of a large follicle or LH surge, to reduce the incidence of cyst formation, and for scheduling of the treatment cycle. These include the use of oral contraceptive pills (OCPs) and luteal administration of E2, progestins, or GnRH antagonists. To the best of our knowledge, there are no RCTs addressing pre-treatment regimens in freeze-all cycles.

While oral contraceptive pills have been extensively used for synchronization of donor and recipient menstrual cycles, the

actual effects of OCPs on COS outcome in donors have been rarely studied. In oocyte donors undergoing COS using a long GnRH-agonist protocol, pre-treatment with a contraceptive vaginal ring resulted in a higher extent of ovarian suppression with significantly lower peak serum E2 levels and lower numbers of oocytes retrieved, but also a lower rate of severe OHSS [23]. The steroid composition of the OCP might also be important as evidenced by a retrospective analysis of oocyte donors undergoing COS with or without OCP pre-treatment [24]. Oocyte yields among donors who utilized higher androgenic OCPs were lower than either donors using no OCPs or those using anti-androgenic OCPs. Thus, the choice of OCP for cycle pre-treatment might also require careful consideration.

The most recent meta-analysis evaluating the effect of OCP pre-treatment on COS outcome in GnRH-antagonist cycles found that the live birth/ongoing pregnancy rate was lower than without pre-treatment (6 RCT, OR 0.74, 95% CI 0.58–0.95, 1335 women). There were no differences between the groups in OHSS rates or the number of oocytes [25]. Currently, there is no clear explanation for the lower live birth/ongoing pregnancy rates in fresh cycles with OCP pre-treatment, and it is unknown whether it is the result of an adverse effect of the OCP on endometrial receptivity or on oocyte competence. Since OCP pre-treatment is common in autologous and donor egg cycles, the effects of OCP pre-treatment on COS for freeze-all cycles call for further research.

Oral progestins such as norethisterone, hydrogesterone (DYG), and medroxyprogesterone acetate (MPA) are commonly used for cycle programming for a variety of indications, including scheduling of COS in order to avoid weekend and holiday oocyte retrievals [26]. Although there are no studies available on progestin pre-treatment in freeze-all cycles, in a recent meta-analysis [25] there was insufficient evidence to determine any differences in rates of live birth or ongoing pregnancy in the GnRH-antagonist protocols (1 RCT, OR 0.67, 95% CI 0.18–2.54, 47 women) and there was no difference between the groups in rates of live birth/ongoing pregnancy in GnRH-agonist protocols (2 RCT, OR 1.35, 95% CI 0.69–2.65, 222 women). There was insufficient evidence to determine whether pre-treatment with progestin resulted in a difference between the groups in the mean number of oocytes retrieved.

Pre-treatment with E2 in the luteal phase of the preceding cycle is often used for improved synchronization of follicular cohort in GnRH-antagonist cycles. Although there is no data available on E2 pre-treatment in freeze-all cycles, in a recent meta-analysis on fresh autologous cycles [25], significantly more oocytes were retrieved following E2 pre-treatment compared to no intervention (2 RCTs, MD 2.23, 95% CI 0.71–3.75, 139 women), with no difference between the groups in the ongoing and live birth rates.

Summary

The potential negative impact of routine use of OCP pre-treatment in COS needs to be further evaluated and carefully balanced with the practical and medical benefits of treatment scheduling (spreading workload for the IVF centre in order to avoid weekend oocyte retrievals and embryo biopsies, and for synchronization during donor egg cycles). Although evidence regarding freeze-all cycles is lacking, the current best estimate is that the advantages of hormone pre-treatment outweigh the drawbacks, and therefore it can be included in COS regimens for freeze-all cycles.

Preferred timing of initiating controlled ovarian stimulation

New evidence suggests that COS may be initiated any time throughout the menstrual cycle, and not necessarily in the early follicular phase. It was demonstrated that two to three “waves” of antral follicles develop in a cyclic manner during the same menstrual cycle [27, 28]. The possibility to stimulate the growth of antral follicles coming from different waves allowed the emergence of new concepts in assisted reproductive techniques (ART): “random start,” in which COS is started any time throughout the cycle, and “Double/Dual stimulation” (DuoStim), in which two stimulations and oocyte retrievals are performed on the same cycle. In both options, freeze-all is inherent in the treatment protocol.

Luteal phase and “random start” stimulation

A meta-analysis [29] of eight retrospective and prospective cohort studies ($n = 338$) compared treatment outcome after luteal phase and follicular phase stimulations. Cycles initiated in the luteal phase were slightly longer and required increased amounts of gonadotropins. No differences were noted in the total number of oocytes retrieved. There were slightly more mature oocytes retrieved following luteal phase stimulation, and fertilization rates were significantly higher. No difference was noted in subsequent pregnancy rates after FET. These studies did not report the LBR.

A large retrospective cohort study ($n = 1302$) evaluated the utility of random-start ovarian stimulation protocols in women who desire elective oocyte cryopreservation [30]. Conventional early follicular (control group), late follicular, and luteal phase ovarian stimulation starts were compared. Although the number of total and MII oocytes in the control and random-start groups was similar, the duration of ovarian stimulation and total dosage of gonadotropins administered was higher in the random-start groups. Another retrospective cohort study [31] has compared the results of 708 patients undergoing luteal phase stimulation, 745 patients undergoing mild COS, and 1287 patients undergoing short GnRH-agonist protocol. The numbers of mature oocytes retrieved and top-quality embryos obtained were significantly increased in the luteal phase stimulation group. No significant differences were identified in the implantation rate, pregnancy rate, or live birth and ongoing pregnancy rates per FET cycle in the luteal phase stimulation and mild treatment groups. However, the luteal phase protocol achieved higher implantation rate, pregnancy rate, and ongoing and LBR compared with the short GnRH-agonist protocol.

DuoStim

A prospective study of 43 poor-prognosis patients undergoing DuoStim and PGT-A found no differences in the number of retrieved oocytes, MII oocytes, or biopsied blastocysts per stimulated cycle from follicular versus luteal phase stimulation [32]. No differences were observed in euploid blastocysts rate. Importantly, luteal phase stimulation contributed significantly to the final transferable blastocyst yield, thus increasing the number of patients undergoing transfer per menstrual cycle. Another case-control study has reported the results of 188 poor-prognosis patients undergoing DuoStim and PGT-A [33]. Follicular phase stimulation resulted in significantly fewer oocytes and fewer blastocysts, compared to LPS. The mean euploidy rates per retrieval were similar between follicular and luteal phase stimulations.

Therefore, on average, fewer euploid blastocysts resulted from follicular phase stimulation. Similar ongoing-pregnancy and delivery rates were reported after follicular- and luteal-derived euploid single blastocyst transfers.

Summary

Moderate-quality evidence indicates that luteal phase stimulation or “random-start” stimulation results in similar outcomes as conventional early follicular stimulation and may be valid future options in freeze-all cycles. Random-start stimulation is clearly indicated in onco-fertility patients where time is an important consideration. There is a clear need for RCTs addressing the efficacy, pharmaco-economic, and long-term child health consequences of “random-start” stimulation, before it can be recommended for elective freeze-all cycles. Currently, early follicular start is the recommended approach.

Combination of gonadotropins used in freeze-all cycles

Since the introduction of FSH-only preparations, it has been constantly debated whether or not LH activity should be added to COS regimens, in the form of either recombinant LH (rLH), human menopausal gonadotropin (hMG), or low doses of hCG, in normogonadotropic patients.

Data on combinations of gonadotropins in freeze-all cycles is lacking. There are very few ($n = 3$) RCTs assessing the addition of LH activity in OD cycles. One study [34] has randomly assigned 42 young oocyte donors to receive either rFSH or rFSH plus rLH in GnRH-antagonist cycles. They found a significant increase in MII oocyte rate (80% vs 71%), fertilization rates (83% vs 71%), top-quality embryos (17% vs 3%), and implantation rates (35% vs 15%) in recipients whose embryos originated from donors receiving rFSH plus rLH. Pregnancy rate was not significantly different. Another study [35] randomly assigned oocyte donors to receive either rFSH alone or rFSH plus LH (in the form of hMG) in a long GnRH-agonist protocol. They observed that only in donors with deep suppression of pituitary LH (<1 IU/L) before the beginning of COS, the inclusion of exogenous LH resulted in an increase in the number of mature oocytes and good-quality zygotes and embryos as well as higher implantation rates when compared with stimulation with FSH alone. In an RCT [36] of 1028 oocyte donors undergoing a long GnRH-agonist protocol, donors were assigned to one of three groups: group 1 received only rFSH; group 2 received only highly purified hMG (hMG-HP); and group 3 received rFSH plus hMG-HP. No differences were found among the groups with respect to number of oocytes retrieved or embryo development parameters. Moreover, implantation, pregnancy, and miscarriage rates with the three regimens were similar. Unfortunately, none of the three studies reported the LBR.

A randomized multi-centre trial [37] of 749 good-prognosis women compared the efficacy of hMG-HP and rFSH for COS in a GnRH-antagonist cycles. The CLBR, resulting from the transfer of fresh and frozen-thawed embryos, for a single stimulation cycle was 40% and 38% for women treated with hMG-HP and rFSH, respectively (non-significant).

Summary

There is insufficient data on type and source of gonadotropin formulations that should be included in freeze-all cycles. Since the population undergoing COS for freeze-all is highly heterogeneous (Table 46.1), personalization of treatment seems imperative. At

present, however, individualization of COS is not evidence-based, and no firm recommendations can be given. Until more data becomes available, clinical choice of gonadotropin preparations should depend on availability, convenience, and costs.

Dose of gonadotropins during COS for freeze-all cycles

Animal studies have suggested an adverse effect of ovarian stimulation on oocyte and embryo quality in a dose-dependent manner [38, 39]. Studies in humans are controversial and less conclusive. Two RCTs reported a higher proportion of good morphologic quality embryos following mild versus conventional stimulation regimens [40, 41], and a positive relationship between doses of gonadotropins and aneuploidy rate, in embryos [41] or in granulosa cells [42]. The recently introduced concept of "ovarian sensitivity index" [43], which is the ratio between the oocyte yield and the total dose of FSH used during COS, suggests that the higher this index is, the higher the pregnancy rates. Thus, well-functioning ovaries that display a good response to low doses of gonadotropins also provide the best oocyte and embryo quality. In agreement with this concept, a recent review of 658,519 fresh autologous IVF cycles, has found the gonadotropin dose as an independent factor which correlated negatively with the likelihood of live birth [44].

Recent studies using modern techniques of PGT-A suggest that COS does not increase the embryo aneuploidy rate [45–47]. Barash et al. [47] retrospectively analysed 4064 biopsied blastocysts and reported that euploidy rates within the same age group were not statistically different, regardless of the total dosage of gonadotropins used or the number of eggs retrieved. In a retrospective cohort study [45], it was demonstrated that the degree of exposure to exogenous gonadotropins did not significantly modify the likelihood of aneuploidy in patients with a normal ovarian response to stimulation. Patients requiring prolonged COS had elevated odds of aneuploidy with increasing cumulative gonadotropin dose, a finding which may reflect an increased tendency towards oocyte and embryonic aneuploidy in patients with a diminished response to gonadotropin stimulation. It has been demonstrated that the number of euploid embryos available for transfer increases as the number of oocytes retrieved does [46]. Recently, a retrospective study has demonstrated that high FSH dosing is associated with reduced LBR in fresh but not subsequent FET cycles, suggesting that the endometrium may be adversely affected, probably indirectly, by high dose gonadotropin use in the fresh IVF cycle only [48].

Summary

Although the exact direct effects of high gonadotropin doses on oocyte and embryo quality remain unknown, the likelihood of favourable reproductive outcome in subsequent FET cycles, as long as euploid blastocysts are available [49], supports the administration of high gonadotropin doses if necessary to achieve an adequately large oocyte cohort.

Preferred regimen for pituitary suppression in freeze-all cycles

Protocols including GnRH agonists have been extensively used in IVF since the mid-80s due to lower cancellation rates, an increased number of oocytes retrieved, and higher pregnancy

rates [50]. The later introduction of GnRH antagonists, which causes profound and immediate pituitary suppression, allowed for less aggressive and more patient-friendly protocols. Initial trials comparing GnRH-agonist and GnRH-antagonist cycles reported slightly but consistently lower pregnancy rates when antagonists were used [51]. Later on, however, it has been demonstrated that the use of GnRH antagonist results in similar LBRs compared with the long GnRH-agonist protocol [52], with a concomitant highly significant reduction in the risk for severe OHSS [51–53]. Furthermore, the possibility to use a GnRH agonist for the final stage of ovulation triggering in patients at high risk for OHSS, has virtually eliminated the risk for severe OHSS in patients at risk [53]. During the last decade, GnRH antagonists became the predominant method for pituitary suppression in ART.

GnRH agonist versus GnRH antagonist

To the best of our knowledge, there are no RCTs reporting the CLBR or LBR in freeze-all cycles with respect to the regimen of pituitary suppression used. Some RCTs reported the pregnancy rate or embryo quality. A large meta-analysis [54] included eight RCTs comparing the regimen for pituitary suppression (GnRH agonist versus GnRH antagonist) in OD programs, with a total of 1024 oocyte donors. hCG was used for final oocyte maturation. There was no difference in the ongoing pregnancy rate between GnRH agonists and GnRH antagonists (RR 1.15, 95% CI 0.97–1.36). The duration of stimulation was significantly lower with the GnRH-antagonist protocol. No significant differences were observed in the number of oocytes retrieved, gonadotropin consumption, or OHSS incidence.

A retrospective study [55] of 175 freeze-all cycles has compared the outcome of blastocysts transfer derived from GnRH-agonist versus GnRH-antagonist COS cycles. In the GnRH-agonist group, significantly higher proportion of blastocysts survived the thawing procedure than in the GnRH-antagonist group (86.1% vs 78.5%; $p < 0.01$), but the CLBR did not differ significantly between the groups. A retrospective study of autologous IVF cycles [56] has found that clinical pregnancy rate (CPR) and implantation rate (IR) were significantly lower in GnRH-antagonist compared to GnRH-agonist cycles with fresh embryo transfer, but were similar in FET cycles.

Toftagger et al. evaluated the CLBR as a secondary outcome from an RCT including 1050 women allocated to a GnRH-antagonist or a long GnRH-agonist protocol. In the original study [57], after the first cycle with fresh embryo transfer, comparable LBRs of 22.8% and 23.8% ($P = 0.70$) were obtained for the GnRH-antagonist and GnRH-agonist protocols, respectively, but the incidence of moderate and severe OHSS was significantly lower in the GnRH-antagonist group. Following a minimum follow-up time from the first IVF cycle of two years, CLBR were similar in GnRH-antagonist and GnRH-agonist protocols, 34.1% versus 31.2%, respectively [58]. A recent retrospective study has found that both GnRH agonist and hCG trigger resulted in comparable euploid rates [59].

Progesterone-primed ovarian stimulation (PPOS)

The strong anti-gonadotropic action of progestins leading to the inhibition of ovulation has been widely utilized in the past in the development of hormonal contraception [60–62]. Recently, oral progestins have also been suggested as an efficient method of preventing premature LH surges during COS [63]. Obviously, progestins interference with endometrial receptivity implies a freeze-all strategy. The use of oral progestins for prevention of

premature luteinization was originally introduced in China as a less costly and more patient-friendly method [64]. In a recent study evaluating the cost-effectiveness of ovulation suppression with progestins compared with GnRH analogues in ART cycles, progestins were found to be cost-effective in elective freeze-all cycles [65].

Both MPA and DYG have moderate to strong progestin action and do not interfere with the measurement of endogenous progesterone production [62, 66], which is a practical advantage for monitoring ART cycles. Several studies examined the use of PPOS during COS. In a prospective cohort study, Kuang et al. [64] administered simultaneously gonadotropins and MPA 10 mg daily beginning on cycle day 3. Ovulation was induced with a GnRH agonist or co-triggered by a GnRH agonist and low-dose hCG. A short GnRH-agonist protocol was used in the control group. Viable embryos were cryopreserved for later transfer in both protocols. The number of oocytes retrieved in the MPA group was comparable to those in the controls (9.9 ± 6.7 vs 9.0 ± 6.0), but significantly longer stimulation duration and higher doses of gonadotropins were required. Only one patient in the PPOS group experienced a premature LH surge (0.7%) and none of the patients in both study arms experienced moderate or severe OHSS. No statistically significant differences were found in the subsequent CPR (47.8% vs 43.3%), IR (31.9% vs 27.7%), and LBRs (42.6% vs 35.5%) in the study group and controls, respectively. Hamdi et al. [67] compared an MPA (10 mg daily) with a conventional GnRH-antagonist protocol in 99 women undergoing IVF, and reported a comparable amount of follicles, large follicles, oocytes retrieved, and embryos generated in both groups. None of the patients in both study arms developed a premature LH surge, and subsequent CPR was also comparable for both groups. In a subsequent RCT [68], PPOS using 4 mg or 10 mg of MPA per day was comparable in terms of the number of oocytes retrieved and pregnancy outcome after FET. The administration of 4 mg of MPA per day was sufficient to prevent an untimely LH rise in women undergoing IVF/ICSI treatment. Two recent studies have compared MPA and GnRH antagonists for pituitary suppression in OD cycles [69, 70]. In both studies, there were no cases of premature LH surge. One RCT has demonstrated comparable COS outcome parameters in both study arms, but in a subsequent non-randomized section of the same study, reproductive outcome in oocyte recipients revealed a lower ongoing pregnancy rate in the PPOS group [69]. In contrast, a retrospective study has found superior COS outcomes and a comparable LBR in recipients from the PPOS arm [70].

A recent RCT, that included 516 patients, was conducted to compare DYG (20 mg/d) with MPA (10 mg/d) introduced with gonadotropins from cycle day 3. It found comparable outcomes for both preparations [71]. Oral administration of micronized progesterone (Utrogestan) has also been shown to be an effective alternative for preventing premature LH surges during COS in normal ovulatory women undergoing IVF, with favourable reproductive outcomes in frozen-thawed embryo transfer [72–74]. Finally, a meta-analysis [75] of 22 studies found similar LBR and ongoing pregnancy rate per ET with progestins and GnRH analogues. The euploidy status of embryos from progestin-primed cycles was also similar to that of embryos from conventional stimulation cycles.

In summary, PPOS has been shown to be an effective and feasible strategy for inhibiting a premature LH surge in patients undergoing COS for freeze-all IVF. Notably, a few retrospective studies recently suggested that progesterone elevation may

impair oocyte and embryo quality [76–79]. Currently, although attractive and promising, as the available evidence on PPOS is limited, its exact role and reproductive outcome in freeze-all cycles should be further evaluated in future studies.

Summary

Although there are no RCTs reporting the CLBR in freeze-all cycles using different pituitary suppression protocols, the preceding data suggest that GnRH-antagonist protocols result in comparable outcomes as GnRH-agonist in freeze-all cycles. Due to the medical and practical advantages of GnRH-antagonist protocols, including lowering the risk of OHSS and improved patient convenience, as well as the possibility to use a GnRH agonist for ovulatory trigger, GnRH-antagonist-based regimens are recommended as the protocol of choice for pituitary suppression in freeze-all cycles. PPOS is a promising approach with apparently similar reproductive outcome. However, further studies are needed to ensure no harm is caused to oocyte and embryo quality by the high concentration of progesterone.

Preferred ovulatory trigger in freeze-all cycles

The combination of a GnRH-antagonist protocol and GnRH-agonist trigger to induce final follicular maturation appears to be highly suitable for freeze-all cycles. The GnRH-agonist bolus used in this context not only induces final follicular maturation but also acts as a luteolytic agent and prevents the secretion of vasoactive substances, mainly vascular endothelial growth factor (VEGF), from the corpora lutea, hence almost completely eliminating the risk of OHSS [80].

GnRH-agonist trigger versus hCG trigger

Several studies and a meta-analysis on fresh autologous cycles have reported a significant reduction in pregnancy and live birth rates with a GnRH-agonist trigger as compared with hCG trigger [81–83]. This was later found to be caused by a severely compromised luteal phase, and not by reduction in oocyte or embryo quality [84]. Griesinger et al. [85] demonstrated that the likelihood of a live birth in FET cycles after GnRH-agonist triggering was not impaired.

The Cochrane Collaboration has published a meta-analysis comparing ovulation triggering with GnRH agonist versus hCG in women who received GnRH antagonist for pituitary suppression [86]. This meta-analysis included 17 RCTs, of which 13 studies assessed fresh autologous cycles and four studies assessed donor-recipient cycles. In fresh autologous cycles, GnRH-agonist trigger was associated with a significantly lower LBR, whereas in donor-recipient cycles, there was no difference in the LBR (1 RCT, $n = 212$) or ongoing pregnancy rate (3 RCTs, $n = 372$) between GnRH-agonist trigger and hCG trigger. As expected, the rate of OHSS was minimal among women receiving GnRH-agonist trigger.

Our systematic search yielded four RCTs that compared the results of OD cycles ($n = 461$) in which donors were randomly assigned to GnRH-agonist trigger or hCG trigger. These four studies have reported similar number of oocytes retrieved, implantation rates, and pregnancy rates [87–90], as well as LBRs [88]. Other retrospective studies of oocyte donors reported similar results [91, 92].

Although severe OHSS has been reported following the use of GnRH agonist for the ovulatory trigger in freeze-all cycles

[93–96], it is an extremely rare event, and most cases of severe OHSS following GnRH-agonist trigger are encountered when small doses of hCG are added to support the luteal phase [97–99]. In addition to a significant risk reduction for OHSS, there is also data suggesting that the GnRH-agonist trigger results in improved oocyte competence and embryo quality [90, 100, 101], either due to a different LH activity on final oocyte maturation compared with hCG or to the induction of the FSH surge, because FSH has been independently shown *in vitro* to have a biological role [102–104]. Since the degree and duration of ovarian enlargement following GnRH-agonist trigger are both reduced, ovarian torsion rate has also been reported to be decreased, adding further to treatment safety [105].

Very few studies have been performed to determine the optimal GnRH-agonist trigger dose that will effectively induce oocyte maturation and prevent OHSS [106–109]. Doses commonly used from Leuprolide vary from 1 mg to 4 mg, and for triptorelin from 0.1 mg to 0.4 mg, without robust data favouring specific doses.

Assessment of GnRH-agonist trigger adequacy

Some clinicians might hesitate to use GnRH agonists for triggering final follicular maturation, due to the risk of insufficient trigger. Different parameters were used to define insufficient GnRH-agonist trigger, including low oocyte recovery rate (the number of oocytes collected divided by the number of large follicles), low oocyte maturation rate (the number of mature oocytes divided by the total number of oocytes), failure to retrieve any oocytes (“empty follicle syndrome”), and low serum LH and progesterone levels on day 1 post trigger. The incidence of insufficient GnRH-agonist trigger was reported to be between 0.6% and 5.5% [110–117].

Some parameters were found to be associated with insufficient GnRH-agonist trigger. These include long-term use of oral contraceptive pills [110, 116], low baseline FSH and LH levels [110, 116, 117], high total dosage of gonadotropins required for stimulation [110, 116, 117], and low BMI [117]. It was suggested that in order to assess the efficacy of the GnRH-agonist trigger, a clinician can measure LH levels on day 1 post trigger. LH levels above 12–15 IU/L were found to indicate effective trigger [112, 118]. In case of insufficient trigger, it was suggested that hCG can be administered on day 1 post trigger [112, 117] or even on the day of OPU, if no oocytes were retrieved from one ovary [117, 119], and oocytes can be successfully retrieved 36 hours following hCG administration [120].

Dual trigger

Recently, it has been suggested that co-administration of hCG and GnRH-agonist (dual trigger) for the final stage of ovulation triggering may improve treatment outcome. In theory, GnRH-agonist administration mimics the physiologic FSH surge, which improves the dissociation of the oocyte from the follicular wall and oocyte recovery, promotes the formation of LH receptors in luteinizing granulosa cells, keeps gap junctions open between the oocyte and cumulus cells, and promotes nuclear maturation and cumulus expansion [102–104].

Our literature search yielded eight trials ($n=1778$) randomizing patients to receive either dual trigger or hCG alone. Unfortunately, none of them has examined freeze-all cycles. Four trials used triptorelin 0.2 mg for dual trigger [121–124], two trials used leuprolide 0.5 and 1 mg [125, 126], one trial used Buserelin 0.5 mg [127], and one trial used an FSH co-trigger [128]. Three trials reported higher pregnancy rates in the dual-trigger groups

[123, 126, 127], while the other five reported similar pregnancy rates. Two trials reported the LBR, which was higher in the dual-trigger group [123, 127]. Most trials reported a similar mean number of retrieved oocytes and similar rates of mature oocytes in both study groups. Three trials reported a higher rate of good-quality embryos in the dual-trigger group [122, 126, 127]. These studies examined only fresh embryo transfer cycles. Therefore, when reporting higher pregnancy or LBRs, it may be in part due to improved endometrial receptivity, which is not relevant in freeze-all cycles.

Due to the potential advantages of the dual trigger, it might be attractive to include the dual trigger for ovulation triggering in freeze-all cycles. However, including hCG in the triggering regimen may put many patients at risk for developing severe OHSS, thus losing the unique safety benefit offered by the GnRH-agonist trigger [80]. One way to introduce a dual trigger with an attempt to simultaneously reduce the risk for OHSS would be to give a low dose of hCG concomitant with the agonist trigger [129, 130]. The dual trigger consisting of a standard dose GnRH agonist and a low hCG dose option should be further evaluated for both safety and efficacy. The possibility to use low doses of hCG may be limited by the lack of availability of low-dose hCG formulations, and by the potential catastrophe which may ensue with the inadvertent administration of a full-dose hCG.

Summary

Based on the evidence presented earlier in the chapter, the GnRH-antagonist protocol with GnRH-agonist trigger is currently the preferred protocol for freeze-all cycles. It has the benefit of almost eliminating the risk of OHSS without compromising the treatment outcome. There are contradicting studies regarding the possible benefits of dual triggering. However, studies examining the CLBR after freeze-all cycles are lacking. It should also be noted that adding hCG to GnRH agonist increases the risk of OHSS.

Criteria for ovulation triggering in freeze-all cycles

The criteria for triggering final follicular maturation in GnRH-antagonist stimulation cycles have never been clearly established. Initially, these criteria were adopted from what was practiced in GnRH-agonist cycles. Tan et al. demonstrated that there was no significant importance in the precise timing of hCG administration after pituitary suppression with GnRH agonist, and paved the way for greater flexibility in scheduling oocyte retrievals [131]. Others have challenged this practice for GnRH-antagonist cycles and demonstrated that early and strict timing of hCG administration may increase the probability of pregnancy [132, 133].

In an RCT comparing hCG administration as soon as three or more follicles of ≥ 17 mm were present on ultrasound or two days later in GnRH-antagonist cycles, prolongation of the follicular phase was shown to be associated with decreased ongoing pregnancy rates [132]. Using a model of egg donors, the same group has shown that prolongation of the follicular phase by delaying hCG administration resulted in a higher incidence of endometrial advancement on the day of oocyte retrieval in GnRH-antagonist cycles [134]. In a subsequent RCT by the same group, patients were randomized to receive hCG either as soon as three or more follicles of size ≥ 16 mm were present or only one day later [135]. Earlier triggering resulted in significantly lower progesterone levels on the day of hCG administration, but also significantly less

mature oocytes compared with the late hCG group, without a difference in the probability of pregnancy. This was confirmed by a retrospective analysis of a large RCT [136], allowing some flexibility in reducing weekend oocyte retrievals in GnRH-antagonist protocols. In another RCT [137], patients received hCG when there were three or more follicles ≥ 17 mm in diameter (Group A), one day later (Group B), or two days later (Group C). Women in groups B and C had significantly more mature follicles than women in group A, although the number of oocytes retrieved did not differ. Fertilization rates and embryo quality were comparable between groups. Pregnancies and live births per cycle were non-significantly higher in groups B and C.

It is generally accepted that larger follicles contain better oocytes, and a positive relationship between follicular size and the level of cytoplasmic maturation has been described [138].

Summary

A summary of the preceding studies [132, 134–137] and the results of a meta-analysis of seven RCTs ($n = 1295$) [139] suggest that a modest (one to two days) delay in ovulation triggering in GnRH-antagonist cycles may result in the collection of more mature oocytes, without an adverse effect on implantation and ongoing pregnancy rates.

A further delay of more than two days resulted in lower ongoing pregnancy rates in fresh cycles, mainly through endometrial advancement. If prolongation of the follicular phase mainly interferes with endometrial receptivity but has no adverse effects on oocyte and embryo quality, then it should be allowed in freeze-all cycles. To the best of our knowledge, this has not been directly evaluated in freeze-all cycles, and deserves further exploration.

Consequences of sustained supra-physiologic oestradiol levels in freeze-all cycles

It has been hypothesized that the supra-physiologic levels of E2 in IVF treatments may impair endometrial receptivity [140, 141]. Moreover, high E2 levels were shown to impair trophoblastic invasion, leading to adverse obstetrical outcomes, such as pre-eclampsia and small-for-gestational-age infants [142, 143]. These data originate from fresh autologous embryo transfer cycles and therefore fit well with the concept of freeze-all, where these negative effects on implantation and placentation are believed to be eliminated by the subsequent transfer of frozen-thawed embryos. In addition, there is contradicting evidence regarding the effect of supra-physiologic levels of E2 on oocyte and embryo quality and fertilization rates [144–146].

Our literature search has yielded only five relevant retrospective studies, two examining the results of autologous FET cycles and three examining OD cycles. A retrospective study of 1122 women [147] compared the treatment outcome according to E2 levels on the day of hCG administration. When examining only FET cycles, the implantation rate and pregnancy rate were similar across all groups of E2 levels. A recent retrospective analysis of autologous IVF cycles has demonstrated that patients with high E2 levels recorded prior to hCG trigger had significantly higher numbers of mature oocytes, zygotes exhibiting two pronuclei, cleavage-stage embryos, blastocysts, and vitrified embryos. Following FET, LBR was significantly higher among patients with higher than normal E2 [148]. A retrospective analysis of 330 consecutive fresh OD cycles [149] has found that sustained supra-physiologic E2 levels did not adversely affect the quality of developing oocytes and embryos. On the contrary, elevated

E2 levels were associated with a larger number of oocytes and embryos and high-grade embryos for transfer/cryopreservation. Another retrospective cohort study of oocyte donors [150] found that higher E2 levels were correlated with higher numbers of oocytes retrieved and embryos available for transfer. Pregnancy rate was similar in cycles with high and low E2 levels. Finally, a retrospective cohort study on 366 oocyte donors with normal E2 response and those with a low E2 response, found no differences in oocyte yield, fertilization rate, blastulation rate, percentage of normal embryos on PGT-A, pregnancy outcomes, and follicular fluid steroid profiles [151].

E2 monitoring

In fresh autologous IVF cycles, a Cochrane review found no evidence from RCTs to suggest that combined monitoring by ultrasound and serum E2 is more efficient than monitoring by ultrasound alone with regard to CPRs and the incidence of OHSS [152]. In a prospective observational study [153], it was demonstrated that ultrasound monitoring is sufficient for an adequate follow up of COS in oocyte donors treated with a GnRH-antagonist protocol and triggered with a GnRH agonist. Since there appears to be no adverse effect for high E2 levels on subsequent FET cycles, it remains to be seen whether monitoring of E2 levels should be included in COS for freeze-all cycles.

Summary

Low quality evidence indicates that high serum E2 levels during COS do not impair oocyte and embryo quality, or subsequent FET outcome. Until more data is available, our best estimate is that sustained supra-physiologic E2 levels are not detrimental to oocyte competence.

Consequences of late follicular phase progesterone elevation in freeze-all cycles

Progesterone (P) elevation on the day of hCG administration has been associated with decreased LBRs in fresh IVF cycles [154–157]. This effect has been attributed to impaired endometrial receptivity, and thus freeze-all cycles should be spared and not affected. A large meta-analysis has been published on this subject [157], including 63 studies on 55,199 fresh IVF cycles, nine studies on 7229 FET cycles, and eight studies on 1330 donor/recipient cycles. In fresh IVF cycles, pregnancy rate was decreased in women with P elevation (defined as ≥ 0.8 ng/mL on the day of hCG administration). However, no adverse effect of P elevation on achieving pregnancy was observed in the FET and the donor/recipient cycles.

In a large retrospective study of 1034 freeze-all cycles, P levels on the day of trigger did not affect the live birth rate after subsequent FET [158]. In another retrospective study of 238 patients undergoing freeze-all cycles [159], P levels on the day of trigger did not affect the number of eggs retrieved and the number of euploid embryos. Elevated P values (≥ 1.5 ng/mL) did not affect the live birth rates in the subsequent FET cycle. A recent prospective study [160] randomized patients with P elevation on hCG day to fresh ET or FET. The CPR was higher in the FET group. Therefore, freeze-all and frozen embryo transfer in subsequent cycles seems a good approach in cases of late follicular phase P elevation.

Recently, several retrospective studies have suggested that late follicular phase P elevation might impair oocyte and embryo quality [76–79, 161] as well as cumulative live birth rates [161] in patients with different ovarian response patterns.

TABLE 46.2 Controlled Ovarian Stimulation for Freeze-All Cycles: Summary of Recommendations

Study Question	Recommendation	Level of Evidence ^a
Optimal number of oocytes	High oocyte yields result in higher CLBR.	2b
OCP pre-treatment	Hormonal pre-treatment can be used to improve follicle synchronization and for scheduling purposes.	2b
Timing of initiating ovarian stimulation	LPS and “random-start” stimulation result in similar outcomes as conventional early follicular ovarian stimulation.	2b
Type of gonadotropins	There are no high-quality studies addressing the issues of type of gonadotropin preparations that should be used in freeze-all cycles.	1b
Dose of gonadotropins	The dose of gonadotropins should be individualized and may be increased as necessary in order to reach the target of a large follicle cohort, as there is no evidence of an adverse effect of high gonadotropin dosage on oocyte and embryo quality and ploidy status.	2b
Regimen for pituitary suppression		
GnRH agonist vs antagonist	GnRH-antagonist protocol offers a good combination of efficacy, safety, and convenience, and should be therefore recommended as the preferred therapeutic approach in freeze-all cycles.	1a
PPOS	The role of PPOS has to be further evaluated.	1b
Ovulatory trigger		
GnRH agonist vs hCG	GnRH-agonist trigger offers a good combination of high safety and efficacy, and should therefore be recommended for freeze-all cycles.	1b
Dual trigger	There are no high-quality studies addressing the issues of dual trigger in freeze-all cycles.	1b
Criteria for ovulation triggering	There are no high-quality studies evaluating ovulation triggering criteria in freeze-all cycles. The best estimate is that a moderate delay of two to three days in ovulation triggering may result in the retrieval of an increased number of mature oocytes and might therefore be recommended.	1a
Impact of supra-physiologic E2 levels	There are no high-quality studies evaluating the effects of sustained supra-physiologic E2 levels on the safety and efficacy of freeze-all cycles. Until now, no significant adverse effects have been described.	2b
Impact of progesterone elevation	Good-quality studies suggest that progesterone elevation does not impair the outcome of FET cycles. Recent low-quality data suggesting an adverse effect on oocyte and embryo quality should be further investigated.	2a

Abbreviations: CLBR, cumulative live birth rate; OCP, oral contraceptive pills; LPS, luteal phase stimulation; PPOS, progesterone primed ovarian stimulation; GnRH, gonadotropin releasing hormone; FET, frozen embryo transfer.

^a According to Oxford Centre for Evidence-based Medicine guidelines.

Summary

Late follicular phase P elevation appears to impair endometrial receptivity, but has no detrimental effect on the outcome of subsequent frozen embryo transfers. Thus, the best estimate is that elevated serum P during COS should not lead to cycle cancellation and should not exclude embryo freezing for subsequent warming and transfer. Since the quality of evidence is low, more studies are needed on the possible effects of elevated P levels on oocyte and embryo quality.

Discussion

In this chapter we aimed to present the best available evidence regarding every aspect of COS in “freeze-all” cycles. For most questions, however, there are no available studies specifically addressing freeze-all cycles. Therefore, we relied on studies on OD cycles or consecutive fresh and frozen ET cycles. Table 46.2 presents a summary of recommendations. The level of evidence for each recommendation is provided according to Oxford Centre for Evidence-based Medicine guidelines (Table 46.3).

TABLE 46.3 Level of Evidence According to Oxford Centre for Evidence-based Medicine

Level	Type of Evidence
1a	Systematic reviews of RCTs
1b	Individual RCT
2a	Systematic reviews of cohort studies
2b	Individual cohort study or low quality RCT

Source: Adapted from <https://www.cebm.ox.ac.uk/resources/levels-of-evidence/oxford-centre-for-evidence-based-medicine-levels-of-evidence-march-2009/>.

Abbreviation: RCT, randomized clinical trial.

In recent years, we have witnessed an exponential increase in the use of “freeze-all” strategy for a variety of indications. Once it was recognized that the stimulation and embryo transfer phases can be separated, new options for manipulating ovarian function emerged, which were not possible as long as ovarian stimulation and embryo transfer were coupled in one cycle. We believe that segmented cycles deserve a unique approach, in order to maximize the reproductive outcomes while ensuring patient safety.

References

1. CDC. 2019 Assisted Reproductive Technology Fertility Clinic and National Summary Report. 2021; Available from: <https://www.cdc.gov/art/reports/archive.html>.
2. Chen ZJ, et al. Fresh versus frozen embryos for infertility in the polycystic ovary syndrome. *N Engl J Med.* 2016;375(6):523–33.
3. Roque M, et al. Fresh versus elective frozen embryo transfer in IVF/ICSI cycles: A systematic review and meta-analysis of reproductive outcomes. *Hum Reprod Update.* 2019;25(1):2–14.
4. Zaat T, et al. Fresh versus frozen embryo transfers in assisted reproduction. *Cochrane Database Syst Rev.* 2021;2(2):Cd011184.
5. Maheshwari A, et al. Elective freezing of embryos versus fresh embryo transfer in IVF: A multicentre randomized controlled trial in the UK (E-freeze). *Hum Reprod.* 2022;37(3):476–87.
6. Roque M, et al. Does freeze-all policy affect IVF outcome in poor ovarian responders? *Ultrasound Obstet Gynecol.* 2018;52(4):530–34.
7. Magdi Y, et al. Revisiting the management of recurrent implantation failure through freeze-all policy. *Fertil Steril.* 2017;108(1):72–7.
8. Mizrachi Y, et al. Ovarian stimulation for freeze-all IVF cycles: A systematic review. *Hum Reprod Update.* 2020;26(1):118–35.
9. Steward RG, et al. Oocyte number as a predictor for ovarian hyperstimulation syndrome and live birth: An analysis of 256,381 in vitro fertilization cycles. *Fertil Steril.* 2014;101(4):967–73.
10. Sunkara SK, et al. Association between the number of eggs And live birth in IVF treatment: An analysis of 400 135 treatment cycles. *Hum Reprod.* 2011;26(7):1768–74.
11. Magnusson A, et al. The number of oocytes retrieved during IVF: A balance between efficacy and safety. *Hum Reprod.* 2018;33(1):58–64.
12. Chatziparasidou A, et al. Accumulation of oocytes and/or embryos by vitrification: A new strategy for managing poor responder patients undergoing pre implantation diagnosis. *F1000Res.* 2013;2:240.
13. Cobo A, et al. Accumulation of oocytes: A new strategy for managing low-responder patients. *Reprod Biomed Online.* 2012;24(4):424–32.
14. Maheshwari A, McLernon D, Bhattacharya S. Cumulative live birth rate: Time for a consensus? *Hum Reprod.* 2015;30(12):2703–7.
15. Zhu Q, Chen Q, Wang L, Lu X, Lyu Q, Wang Y, Kuang Y. Live birth rates in the first complete IVF cycle among 20 687 women using a freeze-all strategy. *Hum Reprod.* 2018;33(5):924–929.
16. Ozgur K, et al. Prediction of live birth and cumulative live birth rates in freeze-all-IVF treatment of a general population. *J Assist Reprod Genet.* 2019;36(4):685–96.
17. Cobo A, et al. Six years' experience in ovum donation using vitrified oocytes: Report of cumulative outcomes, impact of storage time, and development of a predictive model for oocyte survival rate. *Fertil Steril.* 2015;104(6):1426–34.e1–8.
18. Ji J, et al. The optimum number of oocytes in IVF treatment: An Analysis of 2455 cycles in China. *Hum Reprod.* 2013;28(10):2728–34.
19. Drakopoulos P, et al. Conventional ovarian stimulation and single embryo transfer for IVF/ICSI. How many oocytes do we need to maximize cumulative live birth rates after utilization of all fresh and frozen embryos? *Hum Reprod.* 2016;31(2):370–6.
20. Polyzos NP, et al. Cumulative live birth rates according to the number of oocytes retrieved after the first ovarian stimulation for in vitro fertilization/intracytoplasmic sperm injection: A multicenter multinational analysis including approximately 15,000 women. *Fertil Steril.* 2018;110(4):661–70.e1.
21. Vaughan DA, et al. How many oocytes are optimal to achieve multiple live births with one stimulation cycle? The one-and-done approach. *Fertil Steril.* 2017;107(2):397–404.e3.
22. Bodri D, et al. Complications related to ovarian stimulation and oocyte retrieval in 4052 oocyte donor cycles. *Reprod Biomed Online.* 2008;17(2):237–43.
23. Martinez F, et al. A prospective trial comparing oocyte donor ovarian response and recipient pregnancy rates between suppression with gonadotrophin-releasing hormone agonist (GnRHa) alone and dual suppression with a contraceptive vaginal ring and GnRH. *Hum Reprod.* 2006;21(8):2121–5.
24. Barad DH, et al. Does hormonal contraception prior to in vitro fertilization (IVF) negatively affect oocyte yields? A pilot study. *Reprod Biol Endocrinol.* 2013;11:28.
25. Farquhar C, et al. Oral Contraceptive pill, progestogen or oestrogen pretreatment for ovarian stimulation protocols for women undergoing assisted reproductive techniques. *Cochrane Database Syst Rev.* 2017;5:CD006109.
26. Fischer R, et al. A quality management approach to controlled ovarian stimulation in assisted reproductive technology: The "Fischer protocol". *Panminerva Med.* 2019;61(1):11–23.
27. Baerwald AR, Adams GP, Pierson RA. A new model for ovarian follicular development during the human menstrual cycle. *Fertil Steril.* 2003;80(1):116–22.
28. Baerwald AR, Adams GP, Pierson RA. Characterization of ovarian follicular wave dynamics in women. *Biol Reprod.* 2003;69(3):1023–31.
29. Boots CE, et al. Ovarian stimulation in the luteal phase: Systematic review and meta-analysis. *J Assist Reprod Genet.* 2016;33(8):971–80.
30. Pereira N, et al. Random-start ovarian stimulation in women desiring elective cryopreservation of oocytes. *Reprod Biomed Online.* 2017;35(4):400–6.
31. Wang N, Wang Y, Chen Q, Dong J, Tian H, Fu Y, Ai A, Lyu Q, Kuang Y. Luteal-phase ovarian stimulation vs conventional ovarian stimulation in patients with normal ovarian reserve treated for IVF: A large retrospective cohort study. *Clin Endocrinol.* 2016;84(5):720–28.
32. Ubaldi FM, Capalbo A, Vaiarelli A, Cimadomo D, Colamaria S, Alaviggi C, Trabucco E, Venturella R, Vajta G, Rienzi L. Follicular versus luteal phase ovarian stimulation during the same menstrual cycle (DuoStim) in a reduced ovarian reserve population results in a similar euploid blastocyst formation rate: New insight in ovarian reserve exploitation. *Fertil Steril.* 2016;105(6):1488–95.e1.
33. Cimadomo D, et al. Luteal phase anovulatory follicles result in the production of competent oocytes: Intra-patient paired case-control study comparing follicular versus luteal phase stimulations in the same ovarian cycle. *Hum Reprod.* 2018;33(8):1442–48.
34. Acevedo B, et al. Luteinizing hormone supplementation increases pregnancy rates in gonadotropin-releasing hormone antagonist donor cycles. *Fertil Steril.* 2004;82(2):343–7.
35. Tesarik J, Mendoza C. Effects of exogenous LH administration during ovarian stimulation of pituitary down-regulated young oocyte donors on oocyte yield and developmental competence. *Hum Reprod.* 2002;17(12):3129–37.
36. Melo M, et al. A prospective, randomized, controlled trial comparing three different gonadotropin regimens in oocyte donors: Ovarian response, in vitro fertilization outcome, and analysis of cost minimization. *Fertil Steril.* 2010;94(3):958–64.
37. Devroey P, Pellicer A, Nyboe Andersen A, Arce JC. A randomized assessor-blind trial comparing highly purified hMG and recombinant FSH in a GnRH antagonist cycle with compulsory single-blastocyst transfer. *Fertil Steril.* 2012;97(3):561–71.
38. Edwards LJ, et al. Effects of recombinant human follicle-stimulating hormone on embryo development in mice. *Am J Physiol Endocrinol Metab.* 2005;288(5):E845–51.
39. Lee ST, et al. Development of a hamster superovulation program and adverse effects of gonadotropins on microfilament formation during oocyte development. *Fertil Steril.* 2005;83(Suppl 1):1264–74.

40. Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. *J Clin Endocrinol Metab.* 2003;88(1):166–73.
41. Baart EB, et al. Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: A randomized controlled trial. *Hum Reprod.* 2007;22(4):980–8.
42. Kaleli S, et al. High rate of aneuploidy in luteinized granulosa cells obtained from follicular fluid in women who underwent controlled ovarian hyperstimulation. *Fertil Steril.* 2005;84(3):802–4.
43. Huber M, et al. Using the ovarian sensitivity index to define poor, normal, and high response after controlled ovarian hyperstimulation in the long gonadotropin-releasing hormone-agonist protocol: Suggestions for a new principle to solve an old problem. *Fertil Steril.* 2013;100(5):1270–6.
44. Baker VL, et al. Gonadotropin dose is negatively correlated with live birth rate: Analysis of more than 650,000 assisted reproductive technology cycles. *Fertil Steril.* 2015;104(5):1145–52 e1–5.
45. Sekhon L, et al. The cumulative dose of gonadotropins used for controlled ovarian stimulation does not influence the odds of embryonic aneuploidy in patients with normal ovarian response. *J Assist Reprod Genet.* 2017;34(6):749–58.
46. Ata B, et al. Array CGH analysis shows that aneuploidy is not related to the number of embryos generated. *Reprod Biomed Online.* 2012;24(6):614–20.
47. Barash OO, et al. High gonadotropin dosage does not affect euploidy and pregnancy rates in IVF PGS cycles with single embryo transfer. *Hum Reprod.* 2017;32(11):2209–17.
48. Munch EM, et al. High FSH dosing is associated with reduced live birth rate in fresh but not subsequent frozen embryo transfers. *Hum Reprod.* 2017;32(7):1402–9.
49. Irani M, et al. Does maternal age at retrieval influence the implantation potential of euploid blastocysts? *Am J Obstet Gynecol.* 2019;220(4):379 e1–e7.
50. Hughes EG, et al. The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: A meta-analysis of randomized controlled trials. *Fertil Steril.* 1992;58(5):888–96.
51. Al-Inany HG, Abou-Setta AM, Aboulghar M. Gonadotrophin-releasing hormone antagonists for assisted conception. *Cochrane Database Syst Rev.* 2006;(3):CD001750.
52. Al-Inany HG, et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev.* 2011;(5):CD001750.
53. Al-Inany HG, et al. GnRH Antagonists are safer than agonists: An update of a cochrane review. *Hum Reprod Update.* 2011;17(4):435.
54. Bodri D, Sunkara SK, Coomarasamy A. Gonadotropin-releasing hormone agonists versus antagonists for controlled ovarian hyperstimulation in oocyte donors: A systematic review and meta-analysis. *Fertil Steril.* 2011;95(1):164–9.
55. Medved R, Virant-Klun I, Meden-Vrtovec H, Tomazevic T. Outcome of frozen-thawed blastocysts derived from gonadotropin releasing hormone agonist or antagonist cycles. *J Assist Reprod Genet.* 2006;23(6):275–9.
56. Bahceci M, et al. Frozen-thawed cleavage-stage embryo transfer cycles after previous GnRH agonist or antagonist stimulation. *Reprod Biomed Online.* 2009;18(1):67–72.
57. Toftager M, et al. Risk of severe ovarian hyperstimulation syndrome in GnRH antagonist versus GnRH agonist protocol: RCT including 1050 first IVF/ICSI cycles. *Hum Reprod.* 2016;31(6):1253–64.
58. Toftager M, et al. Cumulative live birth rates after one ART cycle including all subsequent frozen-thaw cycles in 1050 women: Secondary outcome of an RCT comparing GnRH-antagonist and GnRH-agonist protocols. *Hum Reprod.* 2017;32(3):556–67.
59. Thorne J, et al. Euploidy rates between cycles triggered with gonadotropin-releasing hormone agonist and human chorionic gonadotropin. *Fertil Steril.* 2019;112(2):258–65.
60. Fotherby K. Potency and pharmacokinetics of gestagens. *Contraception.* 1990;41(5):533–50.
61. Phillips A, et al. A comparison of the potencies and activities of progestogens used in contraceptives. *Contraception.* 1987;36(2):181–92.
62. Regidor PA. The clinical relevance of progestogens in hormonal contraception: Present status and future developments. *Oncotarget.* 2018;9(77):34628–38.
63. Massin N. New stimulation regimens: Endogenous and exogenous progesterone use to block the LH surge during ovarian stimulation for IVF. *Hum Reprod Update.* 2017;23(2):211–20.
64. Kuang Y, et al. Medroxyprogesterone acetate is an effective oral alternative for preventing premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation for in vitro fertilization. *Fertil Steril.* 2015;104(1):62–70 e3.
65. Evans MB, et al. Evaluation of the cost-effectiveness of ovulation suppression with progestins compared with GnRH analogs in assisted reproduction cycles. *Reprod Biomed Online.* 2019;38(5):691–8.
66. Sirois J, Fortune JE. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biol Reprod.* 1988;39(2):308–17.
67. Hamdi K, et al. Comparison of medroxyprogesterone acetate with cetrodote for prevention of premature luteinizing hormone surges in women undergoing in vitro fertilization. *Int J Women's Health Reprod Sci.* 2018;6(2):187–91.
68. Dong J, Wang Y, Chai WR, Hong QQ, Wang NL, Sun LH, Long H, Wang L, Tian H, Lyu QF, et al. The pregnancy outcome of progestin-primed ovarian stimulation using 4 versus 10 mg of medroxyprogesterone acetate per day in infertile women undergoing in vitro fertilisation: A randomised controlled trial. *BJOG.* 2017;124(7):1048–55.
69. Begueria R, et al. Medroxyprogesterone acetate versus ganirelix in oocyte donation: A randomized controlled trial. *Hum Reprod.* 2019;34(5):872–80.
70. Yildiz S, et al. Comparison of a novel flexible progestin primed ovarian stimulation protocol and the flexible gonadotropin-releasing hormone antagonist protocol for assisted reproductive technology. *Fertil Steril.* 2019;112(4):677–83.
71. Yu S, et al. New application of dydrogesterone as a part of a progestin-primed ovarian stimulation protocol for IVF: A randomized controlled trial including 516 first IVF/ICSI cycles. *Hum Reprod.* 2018;33(2):229–37.
72. Zhu X, Zhang X, Fu Y. Uteogestan as an effective oral alternative for preventing premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation for in vitro fertilization. *Medicine (Baltimore).* 2015;94(21):e909.
73. Zhu X, Ye H, Fu Y. The utrogestan and hMG protocol in patients with polycystic ovarian syndrome undergoing controlled ovarian hyperstimulation during IVF/ICSI treatments. *Medicine (Baltimore).* 2016;95(28):e4193.
74. Zhu X, Ye H, Fu Y. Use of utrogestan during controlled ovarian hyperstimulation in normally ovulating women undergoing in vitro fertilization or intracytoplasmic sperm injection treatments in combination with A freeze all strategy: A randomized controlled dose-finding study of 100 mg versus 200 mg. *Fertil Steril.* 2017;107(2):379–86.
75. Ata B, et al. Progestins for pituitary suppression during ovarian stimulation for ART: A comprehensive and systematic review including meta-analyses. *Hum Reprod Update.* 2020;27(1):48–66.
76. Huang B, Li Z, Zhu L, Hu D, Liu Q, Zhu G, Zhang H. Progesterone elevation on the day of HCG administration may affect rescue ICSI. *Reprod Biomed Online.* 2014;29(1):88–93.

77. Huang B, Ren X, Wu L, Zhu L, Xu B, Li Y, Ai J, Jin L. Elevated progesterone levels on the day of oocyte maturation may affect top quality embryo IVF cycles. *PLoS ONE.* 2016;11(1):e0145895.
78. Vanni VS, Somigliana E, Reschini M, Pagliardini L, Marotta E, Faulisi S, Paffoni A, Vigano P, Vegetti W, Candiani M, Papaleo E. Top quality blastocyst formation rates in relation to progesterone levels on the day of oocyte maturation in GnRH antagonist IVF/ICSI cycles. *PLoS ONE.* 2017;12(5):e0176482.
79. Racca A, Santos-Ribeiro S, De Munck N, Mackens S, Drakopoulos P, Camus M, Verheyen G, Tournaye H, Blockeel C. Impact of late-follicular phase elevated serum progesterone on cumulative live birth rates: Is there a deleterious effect on embryo quality? *Hum Reprod.* 2018;33(5):860–68.
80. Devroey P, Polyzos NP, Blockeel C. An OHSS-free clinic by segmentation of IVF treatment. *Hum Reprod.* 2011;26(10):2593–7.
81. Humaidan P, et al. GnRHa trigger and individualized luteal phase hCG support according to ovarian response to stimulation: Two prospective randomized controlled multi-centre studies in IVF patients. *Hum Reprod.* 2013;28(9):2511–21.
82. Youssef MA, et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist assisted reproductive technology cycles. *Cochrane Database Syst Rev.* 2011(1):CD008046.
83. Griesinger G, et al. GnRH agonist for triggering final oocyte maturation in the GnRH antagonist ovarian hyperstimulation protocol: A systematic review and meta-analysis. *Hum Reprod Update.* 2006;12(2):159–68.
84. Eldar-Geva T, et al. Similar outcome for cryopreserved embryo transfer following GnRH-antagonist/GnRH-agonist, GnRH-antagonist/HCG or long protocol ovarian stimulation. *Reprod Biomed Online.* 2007;14(2):48–54.
85. Griesinger G, et al. Triggering of final oocyte maturation with gonadotropin-releasing hormone agonist or human chorionic gonadotropin. Live birth after frozen-thawed embryo replacement cycles. *Fertil Steril.* 2007;88(3):616–21.
86. Youssef MA, et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database Syst Rev.* 2014;(10):CD008046.
87. Melo M, Busso CE, Bellver J, Alama P, Garrido N, Meseguer M, Pellicer A, Remohí J. GnRH agonist versus recombinant HCG in an oocyte donation programme: A randomized, prospective, controlled, assessor-blind study. *Reprod Biomed Online.* 2009;19(4):486–92.
88. Galindo A, et al. Triggering with HCG or GnRH agonist in GnRH antagonist treated oocyte donation cycles: A randomised clinical trial. *Gynecol Endocrinol.* 2009;25(1):60–6.
89. Sismanoglu A, Tekin HI, Erden HF, Ciray NH, Ulug U, Bahcecı M. Ovulation triggering with GnRH agonist vs. hCG in the same egg donor population undergoing donor oocyte cycles with GnRH antagonist: A prospective randomized cross-over trial. *J Assist Reprod Genet.* 2009;26(5):251–6.
90. Acevedo B, et al. Triggering ovulation with gonadotropin-releasing hormone agonists does not compromise embryo implantation rates. *Fertil Steril.* 2006;86(6):1682–7.
91. Bodri D, et al. Triggering with human chorionic gonadotropin or a gonadotropin-releasing hormone agonist in gonadotropin-releasing hormone antagonist-treated oocyte donor cycles: Findings of a large retrospective cohort study. *Fertil Steril.* 2009;91(2):365–71.
92. Shapiro BS, et al. Comparison of human chorionic gonadotropin and gonadotropin-releasing hormone agonist for final oocyte maturation in oocyte donor cycles. *Fertil Steril.* 2007;88(1):237–9.
93. Gurbuz AS, et al. Gonadotrophin-releasing hormone agonist trigger and freeze-all strategy does not prevent severe ovarian hyperstimulation syndrome: A report of three cases. *Reprod Biomed Online.* 2014;29(5):541–4.
94. Fatemi HM, et al. Severe ovarian hyperstimulation syndrome after gonadotropin-releasing hormone (GnRH) agonist trigger and “freeze-all” approach in GnRH antagonist protocol. *Fertil Steril.* 2014;101(4):1008–11.
95. Ling LP, et al. GnRH agonist trigger and ovarian hyperstimulation syndrome: Relook at ‘freeze-all strategy’. *Reprod Biomed Online.* 2014;29(3):392–4.
96. Santos-Ribeiro S, et al. Ovarian hyperstimulation syndrome after gonadotropin-releasing hormone agonist triggering and “freeze-all”: In-depth analysis of genetic predisposition. *J Assist Reprod Genet.* 2015;32(7):1063–8.
97. Connell MT, et al. Larger oocyte cohorts maximize fresh IVF cycle birth rates and availability of surplus high-quality blastocysts for cryopreservation. *Reprod Biomed Online.* 2019;38(5):711–23.
98. Seyhan A, et al. Severe early ovarian hyperstimulation syndrome following GnRH agonist trigger with the addition of 1500 IU hCG. *Hum Reprod.* 2013;28(9):2522–8.
99. Iliodromiti S, et al. Consistent high clinical pregnancy rates and low ovarian hyperstimulation syndrome rates in high-risk patients after GnRH agonist triggering and modified luteal support: A retrospective multicentre study. *Hum Reprod.* 2013;28(9):2529–36.
100. Oktay K, Turkcuoglu I, Rodriguez-Wallberg KA. GnRH agonist trigger for women with breast cancer undergoing fertility preservation by aromatase inhibitor/FSH stimulation. *Reprod Biomed Online.* 2010;20(6):783–8.
101. Humaidan P, et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomized study. *Hum Reprod.* 2005;20(5):1213–20.
102. Atef A, et al. The potential role of gap junction communication between cumulus cells and bovine oocytes during in vitro maturation. *Mol Reprod Dev.* 2005;71(3):358–67.
103. Zelinski-Wooten MB, et al. A bolus of recombinant human follicle stimulating hormone at midcycle induces periovulatory events following multiple follicular development in macaques. *Hum Reprod.* 1998;13(3):554–60.
104. Yding Andersen C, et al. FSH-induced resumption of meiosis in mouse oocytes: Effect of different isoforms. *Mol Hum Reprod.* 1999;5(8):726–31.
105. Berkkanoglu M, et al. Risk of ovarian torsion is reduced in GnRH agonist triggered freeze-all cycles: A retrospective cohort study. *J Obstet Gynaecol.* 2019;39(2):212–17.
106. Pabuccu EG, et al. Different gonadotropin releasing hormone agonist doses for the final oocyte maturation in high-responder patients undergoing in vitro fertilization/intra-cytoplasmic sperm injection. *J Hum Reprod Sci.* 2015;8(1):25–9.
107. Vuong TN, et al. Gonadotropin-releasing hormone agonist trigger in oocyte donors co-treated with a gonadotropin-releasing hormone antagonist: A dose-finding study. *Fertil Steril.* 2016;105(2):356–63.
108. Pozzobon C, Lara E, Zito G, Lucco F, Garrigos V, Ballesteros A. Triggering with different doses of gonadotropin releasing hormone (GnRH) agonist in oocyte donor-cycles: A randomized clinical trial (RCT). *Fertil Steril.* 2013;100(Suppl 3):S60.
109. Zarcos SM, et al. Comparison of two different dosage of GnRH agonist as ovulation trigger in oocyte donors: A randomized controlled trial. *JBRA Assist Reprod.* 2017;21(3):183–7.
110. Popovic-Todorovic B, et al. Predicting suboptimal oocyte yield following GnRH agonist trigger by measuring serum LH at the start of ovarian stimulation. *Hum Reprod.* 2019;34(10):2027–35.
111. Lu X, et al. Dual trigger for final oocyte maturation improves the oocyte retrieval rate of suboptimal responders to gonadotropin-releasing hormone agonist. *Fertil Steril.* 2016;106(6):1356–62.
112. Kummer NE, et al. Predicting successful induction of oocyte maturation after gonadotropin-releasing hormone agonist (GnRHa) trigger. *Hum Reprod.* 2013;28(1):152–9.

113. O'Neill KE, Senapati S, Dokras A. Use of gonadotropin-releasing hormone agonist trigger during in vitro fertilization is associated with similar endocrine profiles and oocyte measures in women with and without polycystic ovary syndrome. *Fertil Steril.* 2015;103(1):264–9.
114. Castillo JC, Garcia-Velasco J, Humaidan P. Empty follicle syndrome after GnRHa triggering versus hCG triggering in COS. *J Assist Reprod Genet.* 2012;29(3):249–53.
115. Chen SL, et al. Circulating luteinizing hormone level after triggering oocyte maturation with GnRH agonist may predict oocyte yield in flexible GnRH antagonist protocol. *Hum Reprod.* 2012;27(5):1351–6.
116. Meyer L, et al. Risk factors for a suboptimal response to gonadotropin-releasing hormone agonist trigger during in vitro fertilization cycles. *Fertil Steril.* 2015;104(3):637–42.
117. Chang FE, et al. Assessing the adequacy of gonadotropin-releasing hormone agonist leuprolide to trigger oocyte maturation and management of inadequate response. *Fertil Steril.* 2016;106(5):1093–1100.e3.
118. Shapiro BS, et al. Efficacy of induced luteinizing hormone surge after “trigger” with gonadotropin-releasing hormone agonist. *Fertil Steril.* 2011;95(2):826–28.
119. Blazquez A, et al. Empty follicle syndrome prevalence and management in oocyte donors. *Hum Reprod.* 2014;29(10):2221–7.
120. Ganer Herman H, et al. Prediction, assessment, and management of suboptimal GnRH agonist trigger: A systematic review. *J Assist Reprod Genet.* 2022;39(2):291–303.
121. Schachter M, et al. Can pregnancy rate be improved in gonadotropin-releasing hormone (GnRH) antagonist cycles by administering GnRH agonist before oocyte retrieval? A prospective, randomized study. *Fertil Steril.* 2008;90(4):1087–93.
122. Decler W, Osmanagaoglu K, Seynhave B, Kolibianakis S, Tarlatzis B, Devroey P. Comparison of hCG triggering versus hCG in combination with A GnRH agonist: A prospective randomized controlled trial. *Facts Views Vis Obstyn.* 2014;6(4):203–9.
123. Kim CH, Ahn JW, You RM, Kim SH, Chae HD, Kang BM. Combined administration of gonadotropin-releasing hormone agonist with human chorionic gonadotropin for final oocyte maturation in GnRH antagonist cycles for in vitro fertilization. *J Reprod Med.* 2014;59(1-2):63–68.
124. Eftekhari M, Mojtabaei MF, Miraj S, Omid M. Final follicular maturation by administration of GnRH agonist plus HCG versus HCG in normal responders in ART cycles: An RCT. *Int J Reprod Biomed.* 2017;15(7):429–34.
125. Mahajan N, Sharma S, Arora P, Gupta S, Rani K, Naidu P. Evaluation of dual trigger with gonadotropin-releasing hormone agonist and human chorionic gonadotropin in improving oocyte maturity rates: A prospective randomized study. *J Hum Reprod Sci.* 2016;9(2):101–6.
126. Seval MM, Ozmen B, Atabekoglu C, Sukur YE, Simsir C, Kan O, Sonmezler M. Dual trigger with gonadotropin-releasing hormone agonist and recombinant human chorionic gonadotropin improves in vitro fertilization outcome in gonadotropin-releasing hormone antagonist cycles. *J Obstetr Gynaecol Res.* 2016;42(9):1146–51.
127. Haas J, et al. GnRH agonist and hCG (dual trigger) versus hCG trigger for final follicular maturation: A double-blinded, randomized controlled study. *Hum Reprod.* 2020;35(7):1648–54.
128. Qiu Q, Chen YD, Huang J, Li Y, Chen XL, Yang DZ, Zhang QX. Does FSH surge at the time of HCG trigger improves IVF/ICSI outcomes-a randomized, double-blind, placebo-controlled study. *Hum Reprod.* 2014;29:i345.
129. Shapiro BS, et al. Comparison of “triggers” using leuprolide acetate alone or in combination with low-dose human chorionic gonadotropin. *Fertil Steril.* 2011;95(8):2715–7.
130. Gunnala V, et al. Sliding scale HCG trigger yields equivalent pregnancy outcomes and reduces ovarian hyperstimulation syndrome: Analysis of 10,427 IVF-ICSI cycles. *PLoS One.* 2017;12(4):e0176019.
131. Tan SL, et al. A prospective randomized study of the optimum timing of human chorionic gonadotropin administration after pituitary desensitization in in vitro fertilization. *Fertil Steril.* 1992;57(6):1259–64.
132. Kolibianakis EM, et al. Prolongation of the follicular phase in in vitro fertilization results in a lower ongoing pregnancy rate in cycles stimulated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonists. *Fertil Steril.* 2004;82(1):102–7.
133. Feichtinger M, et al. Weekend-free scheduled IVF/ICSI procedures and single embryo transfer do not reduce live-birth rates in a general infertile population. *Acta Obstetricia Et Gynecologica Scandinavica.* 2017;96(12):1423–29.
134. Kolibianakis EM, et al. Prolongation of follicular phase by delaying hCG administration results in a higher incidence of endometrial advancement on the day of oocyte retrieval in GnRH antagonist cycles. *Hum Reprod.* 2005;20(9):2453–6.
135. Kyrou D, et al. Is earlier administration of human chorionic gonadotropin (hCG) associated with the probability of pregnancy in cycles stimulated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone (GnRH) antagonists? A prospective randomized trial. *Fertil Steril.* 2011;96(5):1112–5.
136. Levy MJ, et al. Is it possible to reduce the incidence of weekend oocyte retrievals in GnRH antagonist protocols? *Reprod Biomed Online.* 2013;26(1):50–8.
137. Morley L, Tang T, Yasmin E, Hamzeh R, Rutherford AJ, Balen AH. Timing of human chorionic gonadotrophin (hCG) hormone administration in IVF protocols using GnRH antagonists: A randomized controlled trial. *Hum Fertil (Camb).* 2012;15(3):134–9.
138. Ectors FJ, et al. Relationship of human follicular diameter with oocyte fertilization and development after in-vitro fertilization or intracytoplasmic sperm injection. *Hum Reprod.* 1997;12(9):2002–5.
139. Chen Y, Zhang Y, Hu M, Liu X, Qi H. Timing of human chorionic gonadotropin (hCG) hormone administration in IVF/ICSI protocols using GnRH agonist or antagonists: A systematic review and meta-analysis. *Gynecol Endocrinol.* 2014;30(6):431–7.
140. Ullah K, et al. Serum estradiol levels in controlled ovarian stimulation directly affect the endometrium. *J Mol Endocrinol.* 2017;59(2):105–19.
141. Arslan M, et al. Cumulative exposure to high estradiol levels during the follicular phase of IVF cycles negatively affects implantation. *J Assist Reprod Genet.* 2007;24(4):111–7.
142. Farhi J, et al. High serum oestradiol concentrations in IVF cycles increase the risk of pregnancy complications related to abnormal placentation. *Reprod Biomed Online.* 2010;21(3):331–7.
143. Imudia AN, et al. Peak serum estradiol level during controlled ovarian hyperstimulation is associated with increased risk of small for gestational age and preeclampsia in singleton pregnancies after in vitro fertilization. *Fertil Steril.* 2012;97(6):1374–9.
144. Valbuena D, et al. Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. *Fertil Steril.* 2001;76(5):962–8.
145. Kosmas IP, Kolibianakis EM, Devroey P. Association of estradiol levels on the day of hCG administration and pregnancy achievement in IVF: A systematic review. *Hum Reprod.* 2004;19(11):2446–53.
146. Ng EH, et al. Oocyte and embryo quality in patients with excessive ovarian response during in vitro fertilization treatment. *J Assist Reprod Genet.* 2003;20(5):186–91.
147. Yu Ng EH, et al. High serum oestradiol concentrations in fresh IVF cycles do not impair implantation and pregnancy rates in subsequent frozen-thawed embryo transfer cycles. *Hum Reprod.* 2000;15(2):250–5.
148. Sarkar P, et al. Does supraphysiologic estradiol level during IVF have any effect on oocyte/embryo quality? A sibling embryo cohort analysis of fresh and subsequent frozen embryo transfer. *Minerva Ginecol.* 2018;70(6):716–23.

149. Pena JE, et al. Supraphysiological estradiol levels do not affect oocyte and embryo quality in oocyte donation cycles. *Hum Reprod.* 2002;17(1):83–7.
150. Bianco K, et al. Effect of estradiol on oocyte development. *Int J Gynaecol Obstet.* 2009;104(3):230–2.
151. Palmerola KL, Rudick BJ, Lobo RA. Low estradiol responses in oocyte donors undergoing gonadotropin stimulation do not influence clinical outcomes. *J Assist Reprod Genet.* 2018;35(9):1675–82.
152. Kwan I, et al. Monitoring of stimulated cycles in assisted reproduction (IVF and ICSI). *Cochrane Database Syst Rev.* 2014;2014(8):CD005289.
153. Castillo JC, et al. Triggering with GnRH agonist in oocyte-donation cycles: Oestradiol monitoring is not necessary during ovarian stimulation. *Reprod Biomed Online.* 2012;24(2):247–50.
154. Venetis CA, et al. Estimating the net effect of progesterone elevation on the day of hCG on live birth rates after IVF: A cohort analysis of 3296 IVF cycles. *Hum Reprod.* 2015;30(3):684–91.
155. Lawrenz B, Labarta E, Fatemi H, Bosch E. Premature progesterone elevation: Targets and rescue strategies. *Fertil Steril.* 2018;109(4):577–82.
156. Fanchin R. Introduction: Continuing story of premature progesterone elevation. *Fertil Steril.* 2018;109(4):561–2.
157. Venetis CA, et al. Progesterone elevation and probability of pregnancy after IVF: A systematic review and meta-analysis of over 60 000 cycles. *Hum Reprod Update.* 2013;19(5):433–57.
158. Boynukalin FK, et al. Elevation of progesterone on the trigger day exerts no carryover effect on live birth in freeze-all cycles. *Gynecol Endocrinol.* 2021;37(4):367–71.
159. Kofinas JD, et al. Is it the egg or the endometrium? Elevated progesterone on day of trigger is not associated with embryo ploidy nor decreased success rates in subsequent embryo transfer cycles. *J Assist Reprod Genet.* 2016;33(9):1169–74.
160. Yang S, Pang T, Li R, Yang R, Zhen X, Chen X, Wang H, Ma C, Liu P, Qiao J. The individualized choice of embryo transfer timing for patients with elevated serum progesterone level on the HCG day in IVF/ICSI cycles: A prospective randomized clinical study. *Gynecol Endocrinol.* 2015;31(5):355–8.
161. Bu Z, et al. Serum progesterone elevation adversely affects cumulative live birth rate in different ovarian responders during in vitro fertilization and embryo transfer: A large retrospective study. *PLoS One.* 2014;9(6):e100011.

Introduction

Assisted reproduction techniques (ART) have undergone a significant expansion in the last decades. Indeed, the number of *in vitro* fertilization (IVF) cycles has multiplied in the United States from 2000 to the present day, with these techniques being responsible for the birth of more than five million babies worldwide, since the first IVF cycle in 1978 [1]. Therefore, it is not surprising that research in the field of assisted reproduction has increased so dramatically in recent years, resulting in numerous advances that have led to improvements in the outcomes of this clinical discipline.

Most of the studies carried out have focused on avoiding pregnancy losses or implantation failures during IVF cycles. These undesirable results have been considerably reduced thanks to the implementation of certain techniques, such as pre-implantation genetic testing (PGT) followed by euploid embryo selection. It has been widely demonstrated that chromosome screening significantly increases implantation and delivery rates, decreasing the number of miscarriages and achieving clinical pregnancies more often [2].

However, about 30% of cases, in which a chromosomally normal embryo is transferred, still do not yield a live birth. Therefore, it has been concluded that PGT fails to ensure successful implantation and ultimately delivery in all cases. Moreover, not even the improvements made concerning endometrial receptivity, stimulation protocols, or *in vitro* embryo culture can provide a satisfactory response to these lost embryos [3, 4].

This has led both gynaecologists and biologists to seek an explanation in immunology, a scientific field often forgotten in clinical practice, but which has proven to be a relevant player in reproduction. In fact, the role of the immune system in pregnancies has been discussed since 1953, when Peter Medawar, a doctor who had been working on transplantation of skin grafts during World War II, demonstrated that graft rejection, observed in some of his patients, had an immunological cause [5].

Medawar's work raised doubts about the mechanisms that allowed the coexistence of two genetically different individuals during pregnancy, without the occurrence of alloimmune rejection processes, that were so common in transplants [6]. For years, the immune system's involvement in pregnancy has been a widely debated and controversial topic among experts. This is due, on one hand, to the enormous number of cells and molecules that interact to orchestrate immune responses; on the other hand, to their study in peripheral blood, rather than in the uterine micro-environment; and, finally, to a misunderstanding of the role of immunology in reproduction, which fuelled the belief that maternal immune inhibition would favour pregnancies by preventing fetal rejection.

Nevertheless, we now know that the function of the immune system goes far beyond this simplistic hypothesis of fetal rejection. It has been claimed that its proper coordination allows

processes necessary for the achievement of a healthy pregnancy. Some of them are the protection of the fetus against possible pathogens and the establishment of a maternal–fetal tolerance status, which allows adequate placentation [3, 4].

Therefore, inhibiting immune functions to avoid supposed rejections would be a mistake when treating patients, since we would be dysregulating a complex biological process conducted to tolerate and support the fetal implantation and its subsequent development [7].

In this chapter, the cellular components of the immune system that have been most extensively related to reproduction—T cells, macrophages, and natural killer (NK) cells—will be presented. Their functions during pregnancy and the impact they may have in ART will be discussed. In the same way, the current knowledge about the management of autoimmunity and infections in pregnancy, as well as the immunomodulatory treatments that have been used so far in IVF cycles will be reviewed.

T cells, macrophages, and inflammatory states in pregnancy

T cells are known to be a type of lymphocyte, the haematologic cells in charge of recognizing and attacking any material that is foreign to the organism. Specifically, T cells differ from the rest of the lymphocytes in the clonally distributed receptor (TCR) present on their surface. This receptor is built during the fetal development of the individual, by somatic gene rearrangement. In this way, central tolerance is achieved, and these cells become the principal biological elements of alloreactivity.

These lymphocytes are essential in the phenomenon of allore cognition and subsequent rejection of an organ or tissue, as can happen in transplants [8]. It begins with the donor's human leukocyte antigen (HLA) being presented and recognized by the recipient's immune cells. HLA molecules are glycoproteins located on most cell surfaces in the body and, due to their polymorphism, they differ from one individual to another.

During immune responses, HLA antigens can be recognized directly on the surface of foreign cells, or they can be presented by the recipient's antigen-presenting cells (APCs). In one way or another, HLA type I molecules bind to the recipient's CD8+ T cells, which are capable of attacking the foreign graft, and HLA type II molecules are recognized by the recipient's CD4+ T cells. These last cells do not directly damage the transplanted material but promote inflammatory processes, by activating macrophages, while conducting the synthesis of antibodies by B cells. Activation of macrophages results in phagocytosis of any foreign body in the organism and promotion of inflammatory states [9].

This is the way the organism responds physiologically to infectious and non-infectious agents, inducing inflammatory processes. It has been claimed that the establishment of a healthy pregnancy relies on a strict regulation of maternal immune function, ensuring maternal tolerance and an equilibrium between

pro- and anti-inflammatory states, vital aspects for both natural pregnancies and those resulting from ART.

A dysregulation in the maternal immune system could have a negative impact on embryo implantation and placentation. This dysregulation can originate in negative signals leading to lack of activation of maternal-fetal tolerance and impaired embryo implantation or placentation; hyperactivity leading to an increased inflammatory uterine environment and damage on the trophoblast cells; a combination of these processes occurring sequentially.

With all the cascade of recognition orchestrated by the immune system, the question arises as to how it is possible that, during pregnancy, T cells coexist with non-self HLA molecules in the uterine environment without aberrant inflammatory phenomena being observed. Scientists in this field have been working tirelessly to provide some answers to this paradoxical situation [8].

Immune inhibition at the maternal-fetal interface

Some explanations for this unknown are to be found in the genuine cellular and molecular arrangement located at the maternal-fetal interface. Fetal somatic cells are normally completely separated from the maternal immune system by the placental trophoblast barrier, tissue that develops its own mechanisms to avoid immunological recognition. For example, the villous syncytiotrophoblast lacks HLA I and II receptors that activate the maternal immune system through T cells. Likewise, the extravillous trophoblast (EVT), the outermost layer of the future placenta, presents only HLA type I molecules, thus avoiding recognition by CD4+ T cells.

Added to this is the fact that the set of type I molecules expressed in the EVT is not the typical observed in the rest of the organism [10]. The subtypes present are HLA-C, HLA-E, and HLA-G; the latter two being monomorphic, i.e. stable between individuals, which prevents them from being recognized as foreign by the maternal immune system. In addition, HLA-G ligands have a high binding affinity for leukocyte immunoglobulin-like receptors B (LILRB), which are expressed by APCs and macrophages in the decidua, and have been associated with the inhibition of immune responses [11].

Simultaneously, trophoblastic cells contain molecules, such as Fas ligand and indoleamine 2,3-dioxygenase (IDO), with similar inhibitory functions. The interaction of these molecules with T cells promotes their apoptosis, thus decreasing the number of potentially cytotoxic cells in the uterus.

It is thought, therefore, that such inhibitions and lack of T cell activation at the trophoblast-decidua level could be contributing to avoid an immunogenic inflammatory response and, consequently, allow the maintenance of a micro-environment of immunological tolerance during pregnancy [7, 9].

As can be seen, numerous mechanisms have been described to prevent the recognition and generation of alloreactive lymphocytes at the decidual level. In addition, certain forms of autoregulation of the immune system during pregnancy have also been observed in murine models: a decrease in the migration of APCs to the lymph nodes, a reduced number of effector T cells in the decidua due to the silencing effects of environmental cytokines, and the global effects that high levels of progesterone have on the immune system in general, and, on T lymphocytes in particular [12]. In this sense, although translation to humans is difficult, the results of this work also contribute to highlight the role of immunology in pregnancy and the modulations and adaptations required in this state of mother-embryo genetic duality.

Inflammation and immune balances

The presence of a specific type of lymphocyte, T helper (Th1, Th2, and Th17), at the decidual level seems to be essential for the immune adjustments during pregnancy. These Th cells are characterized by expressing the CD4 receptor on their surface and by the different patterns of cytokine production, capable of influencing cellular immunity wherever they act [3].

On the one hand, Th1 lymphocytes secrete proinflammatory cytokines, such as tumour necrosis factor (TNF) α , interferon- γ , and interleukins (ILs) 1, 2, 12, 15, and 18, that activate phagocytosis and increase cytotoxicity. On the other hand, Th2 lymphocytes produce ILs 4, 5, 10, and 13 and granulocyte-macrophage colony-stimulating factor (GM-CSF). They act by favouring the humoral immune response and counteracting the actions of Th1, so their function is considered to be anti-inflammatory.

In the organism there is a Th1/Th2 balance that adjusts according to the immunological challenges faced by the individual, favouring in each case a more or less inflammatory response. Specifically, in the case of pregnancy, the deviation of this cellular balance towards a Th2 dominant response, decreasing the cytotoxic and inflammatory activity of the Th1, has been demonstrated. In this sense, it is believed that it is the increase in progesterone levels that favours the synthesis of anti-inflammatory cytokines (IL-4, IL-10), while, at the same time, decreases the circulation of cytokines related to the Th1 response (IL-2, IL-12, interferon- γ). Moreover, it has been studied that even the presence of an embryo in the uterine micro-environment causes such a deviation of the immune ratio, through the secretion of IL-10 and Transforming Growth Factor (TGF)- β , which also acts by favouring Th2 lymphocyte-mediated responses [3, 13].

Thus, pregnancy takes place in an anti-inflammatory immune state; a pattern that is inherited by the embryo and can be found in the new-born. Therefore, at birth, individuals present a Th2-dominant immune system, which, however, changes with the initial microbial colonization, so that they are also able to generate Th1-mediated responses.

In the context of these statements, it is not strange to wonder whether, indeed, deviations in this immunological balance could be the cause of poor clinical outcomes, both in natural pregnancies and in those achieved by ART. In fact, the disappearance of the Th2 dominance has been observed in anembryonic gestations and cases of recurrent pregnancy loss (RPL). In these studies, a decrease in the interleukins described above, which are necessary for anti-inflammatory responses (IL-10, IL-4), and even the shift of the balance towards Th1 dominance has occurred [14, 15].

However, these imbalances alone cannot be designated as the cause of such undesirable outcomes [3]; they may only reflect gestational loss caused by other unknown factors, or be part of the multiple aspects of a disease, which, if taken together, can have these negative reproductive consequences.

In addition, recent research in immunology no longer gives so much importance to this dichotomous Th1/Th2 model and emphasizes more the role that Th17 cells seem to have, as well as their relationship with T regulators (Tregs).

Th17 is a T helper cell with pronounced proinflammatory actions, through its best-known cytokine, IL-17. On the other hand, Tregs are lymphocytes capable of eliminating alloreactive CD4+ and CD8+ T cells. These last cells have been described in both humans and mice, increasing in frequency in blood during pregnancy and proliferating in the decidua thanks to the action of

certain chemokines produced in that environment, such as TGF- β [12, 16].

In fact, Th17/Treg imbalances (an increase in Th17 and a decrease in Treg subsets) have been observed in women with adenomyosis and endometriosis. This disequilibrium, especially in patients with adenomyosis, has been related to the establishment of a proinflammatory state and immune hyperactivity at the uterine level, leading to reproductive disorders in these individuals [17]. In this regard, an association between this imbalance and early recurrent miscarriage (RM) has been stated by certain authors [18, 19].

Similar deviations towards proinflammatory states have also been described in several disorders that greatly affect fertility; chronic endometritis [20], obesity [21], and polycystic ovarian syndrome (PCOS) [22] are some examples. In these diseases, higher percentages of CD68+ and CD163+ M2 macrophages have been described, as a manifestation of such immunological impairments.

All these facts support the idea of Tregs as necessary cells to achieve a healthy pregnancy, while highlighting the importance of maintaining a moderate level of macrophages and proinflammatory T cells. However, again, these imbalances cannot be established as the sole cause of the poor reproductive outcomes suffered by these patients. This, along with the unavailability of screening tests capable of predicting whether a woman will suffer from these imbalances or not in her future pregnancy, hinders the clinical usefulness of these findings [3].

Immunomodulatory therapies:

Correcting imbalances?

Despite the existing controversy, several attempts have been made to regulate immune responses in pregnant women, so that by correcting the imbalances produced and avoiding possible deviations towards inflammatory and hyperreactive states, the results can be improved. The treatments applied, which will be discussed later in the chapter, are based on the use of TNF- α blockers, intralipids, and intravenous immunoglobulins, intending to recover the dominance towards a Th2 immune state.

To begin with, adalimumab (anti-TNF- α) is an established pharmaceutical biologic product that specifically recognizes and neutralizes the proinflammatory cytokine TNF- α , which, as already explained, is one of those that characterize the cytotoxic response mediated by Th1 lymphocytes. This TNF- α blocker has been used in routine clinical practice for the treatment of inflammatory bowel disease, psoriasis, and rheumatoid arthritis, due to its anti-inflammatory effects [23].

However, its use for women undergoing IVF or intracytoplasmic sperm injection (ICSI) cycles has not yet been adequately investigated, nor are the results of its use in correcting the alleged imbalances between Th1 and Th2 lymphocytes in these patients known. In fact, no randomized controlled trials have been found to support its use, and the research carried out is based on certain observational studies.

Some investigations on the possible advantages of using adalimumab, along with other therapies, in ART patients with Th1/Th2 ratio deviations seem to reveal better clinical outcomes (implantation, clinical pregnancy, live birth rate) in the treated versus control groups [24, 25]. However, we must be extremely careful when interpreting these results, as these research projects present a poor experimental design that makes it impossible to translate these conclusions to routine medical practice. It is,

therefore, necessary to continue studies on the subject before introducing this drug in the usual lines of treatment of IVF or ICSI patients, since there is still not enough evidence from adequately designed research to support its use [23].

In addition to the lack of evidence regarding its usefulness in ART, it is noteworthy that its safety during pregnancy is highly questioned by some publications. Specifically, a systematic review found a trend towards drug-specific harm with increased risk of congenital malformations and preterm birth in infants of women exposed to TNF- α blockers during pregnancy [26].

There is an open debate regarding this subject, with numerous publications that contradict this association [27]. These controversies, together with the relatively long half-life of these drugs, their transplacental transport, and their possible action producing neonatal immunosuppression, which would favour infections, leads many physicians to advise against their use during pregnancy, or at least in the third trimester of gestation [27, 28].

Its use, therefore, should not be contemplated in pregnant women without pathologies and must be studied on a case-by-case basis for those using TNF- α blockers as a treatment for inflammatory diseases, such as psoriasis. These illnesses would also affect pregnancy and the advantages and disadvantages of stopping treatment would have to be determined [27].

Another possible therapeutic approach that has been proposed with the aim of avoiding dominant Th1 immune responses is based on the use of intralipids, fat emulsions, nowadays used for parenteral nutrition, which could act by suppressing the activity of proinflammatory cytokines.

In this regard, only one randomized placebo-controlled double-blinded trial has been conducted. In this study, the authors found no significant difference in the chemical pregnancy between the control group and the intralipid-treated group, although it was noted a borderline significant difference in ongoing pregnancy and live birth in favour of intralipid [29]. However, the power of the research carried out does not allow us to affirm that this difference is not due solely to chance, rather than to the treatment.

In addition, a prospective cohort study, also conducted to test the usefulness of this therapy, had to be stopped early when not a single birth was recorded in the intralipid-treated group, while the live birth rate in the control group was close to 30% [30].

Thus, it is concluded, as with adalimumab, that its use should not yet be introduced into clinical practice, as none of the trials conducted has succeeded in proving the safety and usefulness of this treatment.

The last proposed immunomodulatory therapy that will be reviewed to maintain an adequate Th1/Th2 lymphocyte ratio is the intravenous injection of immunoglobulin G (IVIG). This treatment has proven to be very useful in the fight against certain inflammatory and autoimmune pathologies, including Guillain-Barré syndrome, relapsing inflammatory polyneuropathy, and Kawasaki disease.

In fact, its usefulness is based on the belief that this IVIG is capable of reducing the number of cytotoxic NK cells and B lymphocytes, as well as increasing the number of Tregs. Thus, its use in ART would allow regulating the immune system, diverting the possible Th1 cytotoxic responses towards the more desirable Th2 ones, according to the existing literature [23].

There are, however, not many human trials to ensure its effectiveness in patients undergoing IVF or ICSI cycles. Nevertheless, it is worth noting the conclusion reached by

some authors who studied the use of this and other therapies in the context of ART. According to them, the use of IVIG may be beneficial for selected groups of patients with an increased Th1/Th2 ratio or an abnormally high number of CD56+ CD3-cells [24, 31].

Some of the studies reaching such conclusions, though, present low statistical power. This, along with the fact that most publications fail to find better outcomes associated with the use of IVIG, leads us to believe that, in reality, this therapy is not useful in the ART setting [7].

In this regard, a study conducted including patients with previous miscarriages or implantation failures registered a significantly higher implantation rate in the group treated with IVIG. However, this was not subsequently reflected in the pregnancy rate, which showed no difference between the two groups [32]. In the same way, research to verify the usefulness of IVIG in IVF and egg donation cycles concluded that this treatment was unable to improve reproductive outcomes, suggesting that other parameters, such as embryo quality, were of more major importance than the maternal immunological status [33]. Similar conclusions were published by a subsequent meta-analysis [34], which highlighted the absence of good-quality evidence to support the use of IVIG in ART.

As can be seen, research on this subject has not found any usefulness in the use of this immunomodulatory therapy, failing to improve the clinical results of the cycles. In addition, its high cost and its association with several side effects, such as anaphylactic shock, make its implementation inadvisable [35].

Indeed, certain regulatory entities, such as Human Fertilization and Embryology Authority (HFEA), point out that this and the other immunomodulators discussed in this section (TNF- α blockers and intralipids) are not recommended add-on treatments. In other words, these therapies added to the assisted reproduction cycle are not effective at improving the chances of having a baby for most fertility patients [36].

However, although the available evidence suggests that these immunomodulatory therapies are far from being of any benefit to patients in general, research on the subject has forced physicians to pay attention to immunology as a central aspect of pregnancy, as well as to the immunological imbalances that many patients present. It is equally important to bear in mind that personalized medicine is essential, given that certain therapies may improve clinical results in ART cycles only in selected populations, requiring, therefore, a prior study of each couple to determine the benefits, if any, of applying the immunomodulatory treatments analysed in this chapter.

In conclusion, and according to the preceding, many mechanisms make it possible to establish a maternal–fetal tolerance that ensures the simultaneous coexistence of two genetically different identities. Likewise, inflammation has been shown to be a process that must be strictly controlled to achieve an adequate early placentation and a healthy subsequent gestation.

It is important to highlight the role of Treg cells and the maintenance of appropriate immune balances to avoid pregnancy complications and disorders. In addition, this literature review highlights the need to continue research on possible therapies aimed at favouring appropriate immune responses mediated by macrophages and T lymphocytes, cells that form an essential part of the entire immune machinery that is set in motion to adapt the maternal system to pregnancy, and whose other cellular components will be discussed throughout this chapter.

Natural killer cells: Roles in placentation and immunological compatibility

Natural killer (NK) cells are another type of lymphocyte that, like T cells, has been extensively studied in the context of pregnancy and ART. Their discovery dates to the 1970s, but their evolutionary origin is earlier than that of T lymphocytes [37]. Both cell types are very similar in terms of functions, phenotypes, etc.; the major difference being the absence of the TCR in NK cells. Therefore, these lymphocytes do not present on their surface a receptor obtained from the somatic genetic reorganization for the target recognition of everything foreign to the organism itself.

Despite that, they are able to carry out their immunological function through the HLA ligands, already mentioned. In this sense, it has been established that NK cells recognize and attack all those cells lacking self-HLA class I molecules, this cytotoxic activity being inhibited when they bind to cells expressing the organism's own HLA class I ligands. This kind of action is known as missing self-response and is essential, for example, in the body's defence against viral infections and cancer [8].

Uterine natural killer cells versus peripheral blood natural killer cells

The fact that NK cells have been described as the most abundant lymphoid population in the uterus (70%–90% of lymphocytes), as opposed to their small number in peripheral blood (5%–10% of lymphocytes), suggests their possible involvement in fertility [3]. Along with this distinction in localization, certain differences have been recorded that allow researchers to establish uterine natural killer (uNK) cells as a different population from those found in the bloodstream (pbNK). Indeed, phenotypic differences in terms of the receptors expressed on the surface of these cells, measured by immunohistochemistry, have been confirmed.

It has been determined that uNK cells show a high expression of CD56 and are negative for CD16 ($CD56^{bright} CD16^-$), whereas 90% of pbNK cells express less CD56 and do contain surface CD16 molecules ($CD56^{dim} CD16^+$) [38]. The distinctions go further, and several authors have confirmed that the typically cytotoxic responses generated by pbNK cells, essential in the fight against neoplasia and infections, are hardly seen in uNK cells. Conversely, the latter stand out more for their role in cytokine production [7, 39].

Because of these differences (localization, superficial receptors ...) the measurement of pbNK cells does not necessarily correlate with the number of NK cells found in the uterine micro-environment, and any treatment that might be administered in the context of ART based on these blood measurements would not have been adequately introduced. In fact, it has not been possible to establish a relationship between the quantity of uNK cells and those of peripheral blood, and even different patterns of variation have been recorded in these populations in the organism, depending on specific conditions [3, 40].

Actually, there is a fluctuation in the total number of NK cells, according to the immunological status of the patient, the presence of infectious agents, stressful situations, exercise ... but what is especially remarkable is the existing dependence between the number of uNK cells and the phase of the menstrual cycle, a phenomenon not observed for their equivalents in peripheral blood [4, 41].

In this regard, during the proliferative phase, there are low levels of uNK cells, whose quantity increases after ovulation,

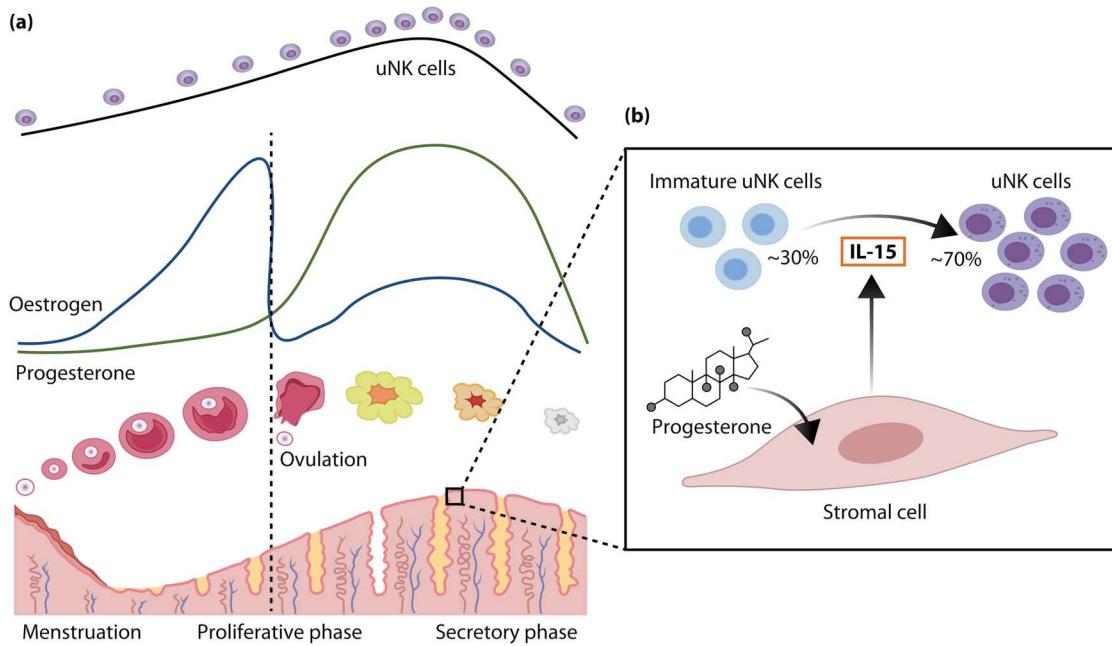


FIGURE 47.1 (a) uNK cells throughout the menstrual cycle. The increase this population suffers during the proliferative phase can be observed, along with the subsequent reduction if fertilization does not occur. (b) Proposed mechanism of progesterone-dependent uNK cell maturation and proliferation [7].

reaching a maximum in the late secretory phase (Figure 47.1a). This increase is maintained, if fertilization occurs, during the first weeks of gestation and declines once the trophoblast has completed invasion, approximately around week 20 of pregnancy. These cyclic changes and the fact that the concentration of uNK is much lower in the endometrium of premenarchial and post-menopausal women led researchers in the field to conclude that such fluctuations could be explained by hormonal factors [7, 42].

Progesterone has been established as the hormone capable of controlling these changes in the levels of uNK cells, since the variations measured in this hormone are reflected in the same sense in the number of such lymphocytes in the endometrium. However, such hormonal control must be indirect, since uNK cells lack progesterone receptors (PR) [43]. The proposed mechanism, therefore, is that it is the stromal cells, which do have PR, that react to increases in progesterone levels and, in response, release IL-15 (Figure 47.1b). This cytokine favours the proliferative activity of the uNK cells and, therefore, has been pointed out as the direct cause of the variations observed in this lymphoid population [8, 44].

Further evidence of this pathway is found in the fact that, if fertilization does not occur, the degeneration of the corpus luteum produces a drop in progesterone and, consequently, in IL-15, and a marked decrease in the number of uNK cells is observed. Therefore, IL-15 is the central cytokine responsible for the multiplication and differentiation of these lymphocytes, thus allowing them to carry out their function, which will be discussed later, during the menstrual cycle and the first trimester of pregnancy [45].

The origin of uNK cells

However, even though this question has been resolved, many unknowns surrounding uNK cells remain. The first to be

considered focuses on the origin of this specific population of lymphocytes. Many authors have repeated the question: Where do uNK cells come from? Do they already exist in the uterus, or do they migrate there from elsewhere in the body in response to hormonal changes?

Many plausible hypotheses have arisen in this regard. For example, some researchers point to the possibility that it is a local effect; in other words, that a cellular differentiation at the uterine level converts certain cells or precursors into the lymphoid population. This phenomenon could happen from pbNK cells resident in the uterus, which would be transformed into uNK cells through the supposed action of TGF- β . This cytokine, synthesized by stromal cells, would promote the change from pbNK CD16⁺ to uNK CD16⁻ cells. Likewise, there is a possibility that the switch to uNK cells occurs not only from pbNK cells but also from undifferentiated progenitor cells, expressing CD34, located in the decidual tissue [38, 46].

The other theories in this regard defend the migration of pbNK cells or haematopoietic stem cells from the bloodstream to the uterus. This recruitment would be regulated by cytokines, as would be the subsequent differentiation of these precursors to uNK cells, once established in the uterine micro-environment. In this location, the presence of IL-15 and other interleukins would favour an optimal atmosphere for conversion to uNK cells and subsequent proliferation [7, 46, 47].

As described, several hypotheses have arisen in an attempt to explain the origin of this lymphocyte population, predominant in the uterus and whose levels vary according to the menstrual cycle and the event of pregnancy. However, it is still unknown whether this differentiation process is completely local; whether the precursors are recruited from the blood or come from a pre-existing uterine population; or even whether it is a combination of both phenomena [7].

The lack of knowledge regarding various aspects of uNK cells is of concern, considering their high presence in the uterine micro-environment and the growing awareness of the important role that immunology plays in the achievement of a full-term pregnancy.

uNK cell functions in the context of pregnancy:

From rejection theories to important roles

in angiogenesis and EVT invasion

As explained before, cytotoxic activity in uNK cells is hardly seen; especially when compared to that of pbNK cells. Nevertheless, for a long time, both populations were referred to as NK cells in a generic way, combining their characteristics and functionalities. Hence, several researchers and clinicians were influenced by the ideas of cell toxicity implicit in the name “natural killer” and proposed them as a possible mechanism by which the maternal organism could attack the embryo, rejecting it [4, 7].

These thoughts were fuelled when several researchers published the results of uNK and pbNK cell measurements in women undergoing ART. According to these articles, a higher number of such cells were observed in patients with RM and other reproductive disorders compared to the rest [48, 49]. It was hypothesized, therefore, that an exaggerated activation of the immune system of these women, in terms of increased potentially dangerous NK cells, is what could be causing the gestational losses suffered.

Despite systematic reviews soon appearing that found no clear correlation between the quantity of these cells and reproductive outcomes [50, 51], this aspect had long been one of the most controversial of the involvement of immunology in reproduction, until further study of uNK cells elucidated their actual role in the development of pregnancy to be quite different from these hypothetical rejections.

In fact, finally, it was confirmed that there is no evidence of embryonic immunological rejection phenomena caused by NK cell populations. Nothing could be further from the truth; similar to what happened with T cells, these cellular components seem to be one of the fundamental pillars for the achievement of maternal–fetal tolerance, as well as for the adequate formation of the placenta, which allows embryonic nourishment until delivery [7, 52]. Therefore, the increased NK cell number observed in some of the patients with RM could be affecting the correct gestational development, not because of the supposed immunological attack on the embryo, but because the processes regulated by these cells would not be carried out in an adequate manner, since the cellular elements in charge of them are out of the normal range.

uNK cells are the most abundant lymphocytes during the first trimester of gestation. This is consistent with their assigned role as modulators of angiogenesis and the establishment of adequate placentation blood supply [53, 54]. This process is carried out through the modification of the spiral arteries, branches of the uterine artery that will be responsible for supplying nutrients and oxygen to the embryo. In this way, the uNK cells ensure embryo survival by multiplying by 100 the blood flow that reaches the uterus through these arteries [45, 53].

The **remodelling of the spiral arteries** at the onset of gestation involves a series of processes: vessel dilation, increased permeability, progressive loss of endothelial cells, separation of the VSMC (vascular smooth muscle cells), and endovascular invasion of the EVT. All these changes would be controlled by uNK cells [55].

However, these cells not only play important roles during pregnancy but are also necessary for **proper endometrial homeostasis**. In this sense, women with heavy menstrual bleeding present

an altered vascular maturation of the endometrium, linked to a dysregulation of uNK cells. The endometrial cycle requires a fluctuation of such cells, as just explained, reaching a maximum during the window of implantation. Any dysregulation in this cycling process leads to loss of homeostasis and the occurrence of irregularities, such as the heavy menstrual bleeding already mentioned [56].

Therefore, it can be determined that uNK cells play an important role in the female fertility scenario, both in pregnancy and in general homeostasis processes; actions that they carry out through paracrine signals. Indeed, the expression of certain angiogenic factors (VEGF-C, Ag1, Ang2 ...) by these cells has been observed in the non-pregnant endometrium and, above all, during the first weeks of gestation. Through these secretions, angiogenesis processes are increased, and remodelling of the spiral arteries occurs, which ensures an adequate supply of blood flow from the mother to the embryo. These are critical steps in pregnancy, especially in its earliest stages [7, 55]. Certain polymorphisms in the genes encoding some of these angiogenic factors have been linked to idiopathic RM, demonstrating the importance of this vascular remodelling process, and the need for all the molecules and cells involved to act appropriately to achieve the correct blood flow for a healthy pregnancy [57].

Likewise, uNK cells are responsible for **controlling trophoblastic invasion**. Initially, it was believed that these cells promoted EVT invasion through direct cytotoxic action. However, it is now known that uNK cells, despite their name, inherited from their blood homologs, show hardly any cytotoxicity, making this approach erroneous [55].

Conversely, its function is to regulate this process, preventing poor or excessive invasive activity by trophoblastic cells, through the secretion of a series of cytokines. This could explain episodes of ectopic pregnancy or adhesion of the embryo to the scar tissue of a previous caesarean section, where exaggerated growth and uncontrolled invasion occur. On these occasions, the absence of decidua and, consequently, of the cells that populate it (such as uNK cells) is what prevents the invasion process from being controlled and, with it, a regulated construction of the placenta [7].

The cytokine production pattern of uNK cells has been extensively studied and it has been demonstrated *in vitro* that some of the products secreted by these cells, such as TNF- α , TGF- β 1, and IFN- γ , are able to inhibit trophoblast invasion [58, 59]. Similarly, researchers in the field described that co-culture of uNK cells with placental explants taken at 12–14 weeks gestational age induced the invasive activity of the EVT [60].

The latter, however, was not observed when coculture was performed with explants of earlier gestational ages (8–10 weeks). This is because the secretions produced by uNK cells appear to change as pregnancy progresses, producing specific factors appropriate to each gestational age. In this sense, the synthesis of angiogenic factors has been described predominantly during the first moments of pregnancy (up to week 10) and, subsequently, a change of secretions towards cytokines for the regulation of trophoblastic invasion would take place (**Figure 47.2**).

The mechanism for the switch between these two functions and the differing angiogenic growth factor and cytokine profiles is not yet clear. However, these findings demonstrate the fine control that is required during the processes that determine adequate placentation, since the future of the pregnancy will depend on them. Angiogenesis and the invasive process of EVT must be tightly regulated and take place at the right time, which is at 8–10 weeks of gestational age for the former and from 12 weeks

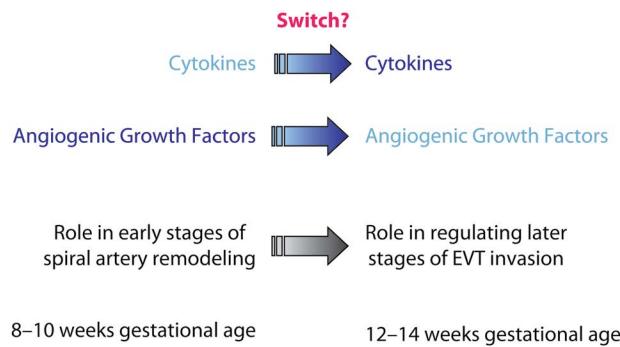


FIGURE 47.2 Schematic of the differences in uNK cells functions and secretions with gestational age. Since 12 weeks of pregnancy, these cells start to produce more cytokines implicated in the regulation of EVT invasion and reduce the secretion of angiogenic factors [55].

onwards for the latter. The occurrence of any of these phenomena outside the established timeframe, as well as exaggerated or insufficient angiogenesis and/or invasion, may compromise the progression of the pregnancy and may lead to undesirable results, such as preeclampsia, RM, or RIF [7, 52, 55].

The complex cellular and molecular interactions that occur at the maternal–fetal interface ensure the proper development of these early pregnancy processes. There are an enormous number of factors involved as well as interactions at different levels (molecular, cellular, tissue, etc.) with a great diversity of genetic combinations that make research in this area extremely difficult and give an idea of the complexity of the immune system and the important role played by each of its components in the proper achievement of pregnancy.

Despite the vast diversity of biological aspects involved, the interactions that occur between uNK cells and trophoblastic cells, as explained before, have proven to be one of the most important in the correct development of angiogenesis and placental tissue invasion, and have constituted an essential field of study in the immunological aspects of reproduction.

Maternal–fetal interactions: KIR/HLA-C combinations and reproductive consequences

The main interactions between uNK cells and EVT occur through the HLA molecules (Figure 47.3). Specifically, HLA-G, whose expression by trophoblastic cells has been widely investigated, binds to members of the LILR family and killer-cell immunoglobulin-like receptors (KIR). Certain clinical parameters, such as placental and fetal weight, as well as the development of pre-eclampsia, have been related to this class of HLA molecules and their interactions in the decidua. Likewise, they have been associated with the establishment of maternal–fetal tolerance [61, 62].

On the other hand, HLA-E molecules find their receptors on CD94/NKG2 heterodimers on the surface of uNK cells (Figure 47.3). The functions of these ligands may be related to the release of cytokines by this lymphocyte population, favouring and ensuring a sufficient nutritional supply for fetal growth. Similarly, HLA-F binds to NK receptors ILT2 and ILT4, but its function has not yet been elucidated [7, 63, 64].

Finally, the last type of HLA molecules expressed by EVT, HLA-C, interact with uNK cells through KIR receptors [61]. These junctions are the ones that have attracted the most attention of researchers, firstly, because of the high degree of genetic polymorphism that they present and, secondly, because of the impact that the different possible genetic combinations seem to have on reproductive outcomes.

KIRs are encoded by a group of highly polymorphic genes located on human chromosome 19q13.4. They have been classified depending on the number of immunoglobulin-like extracellular domains into two groups: KIR2D and KIR3D, encoding two and three of such domains successively. In the same way, a sub-classification has been established depending on the length of the cytoplasmic tail encoded by each gene (Figure 47.3), which can be long (KIR2DL and KIR3DL) or short (KIR2DS and KIR3DS) [65, 66].

However, the most relevant classification for the progression of this chapter that has been most used in the scientific field is based on the functional aspects of these receptors. In this regard, the more than 1110 KIR alleles have been divided into two categories—**KIR A, inhibitors** and **KIR B, activators**—according to

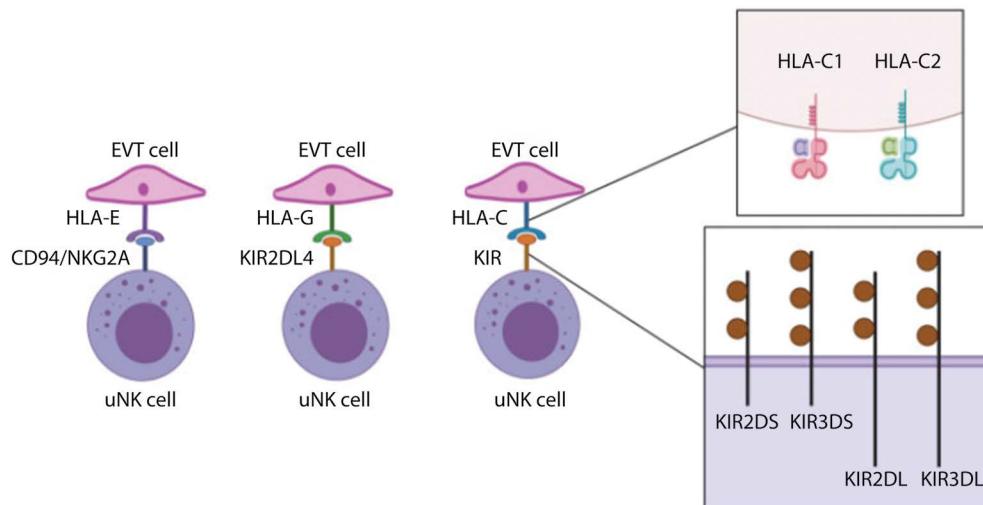


FIGURE 47.3 Interactions between uNK cells and HLA ligands from EVT cells. Isoforms of HLA-C are also presented, as well as KIR classification, depending on the number of extracellular domains and tail length [7].

their ability to regulate the behaviour and functions of the uNK cells that express them [67]. In addition, a short cytoplasmic tail has been associated with activating KIR receptors, while a long cytoplasmic tail has been described in inhibitors [68].

Following these classifications, it has been decided to establish haplotypes for these receptors according to the type and number of each of these genes. Thus, haplotype A is determined by the presence of the seven KIR3DL3-2DL3-2DL3-2DL1-2DL4-3DL1-2DS4-3DL2 genes.

Of these, only 2DS4 is considered activator, but presents a mutation which makes it non-functional. Any other combination of the KIR loci determine the haplotype B. Depending on the inherited haplotypes, each person's KIR genotype could be AA (no activating KIR), AB, or BB (different activating KIR) [7, 69]. Another classification exists, also used in clinical practice, which only distinguishes between KIR AA and KIR Bx based on the absence or presence of the activating KIR, respectively.

The ligands for these KIR receptors, the HLA-C molecules expressed by EVT cells, are similarly classified into two groups, according to the existence of a dimorphism in position 80 of the $\alpha 1$ domain: HLA-C1, if the amino acid occupying this position is asparagine, and HLA-C2, if it is lysine [70]. In accordance with these distinctions, different affinities of these allotypes to their KIR receptors have been observed. Thus, HLA-C1 is the ligand of the inhibitory receptors KIR2DL2 (haplotype B) and KIR2DL3 (haplotype A), whereas HLA-C2 is the ligand of the activating receptors KIR2DS1 (haplotype B) and the inhibitory receptors KIR2DL1 (haplotype A). It has been observed that the binding between HLA-C2 and the inhibitory KIRs is of much greater strength than the interaction mediated by the C1 allotype, leading to stronger suppressive effects on uNK cells [71].

During pregnancy, the combination of maternal KIR and partially paternal fetal HLA-C alleles appears to affect reproductive outcomes, with certain HLA/KIR bindings being associated with a higher risk of pregnancy disorders. Several studies have

established that women with KIR AA genotype (lacking activating KIR) are the most likely to suffer from pre-eclampsia and RM. Moreover, if the maternal homozygous KIR AA genotype coincides with the fetus having more HLA-C2 genes than the mother, the risk of pre-eclampsia, RM, and also fetal growth restriction (FGR) is further increased [69, 72, 73].

The underlying cause of these observations seems to lie in the exaggerated inhibition that these HLA-C2s produce on uNK cells, with the KIR2DL1 receptor proposed to be the most implicated in these negative effects, due to the high affinity with which it binds to its HLA ligand. Conversely, the presence of the KIR2DS1 receptor, haplotype B, has been established as protective against these situations, by binding to C2 allotype and promoting activation of uNK cells, which would compensate for the inhibition produced by KIR2DL1.

Circumstances of increased inhibitory effect on uNK cells would lead to a decrease in the production of cytokines and angiogenic factors, which, in turn, would prevent the adequate placentation required for the normal course of pregnancy. It is the poor trophoblastic invasion (Figure 47.4) that would occur in this situation that is related to the occurrence of RM, pre-eclampsia, and FGR [7, 73, 74].

These scenarios may be observed more prominently during cycles of assisted reproduction treatments. These involve situations such as double embryo transfer (DET), which, although progressively less common, still exists; and oocyte donation protocols. In these cases, the higher number of non-self-antigens (HLA-C) presented to the mother's uNK cells KIR receptors would increase the likelihood of the adverse effects that appear to be associated with certain HLA/KIR combinations.

In fact, the literature describes higher maternal morbidity (pre-eclampsia, FGR ...) and preterm birth in oocyte donation pregnancies compared with ART pregnancies with the patient's own oocytes [75, 76]. Although it is true that part of these complications can be explained by advanced maternal age, which is

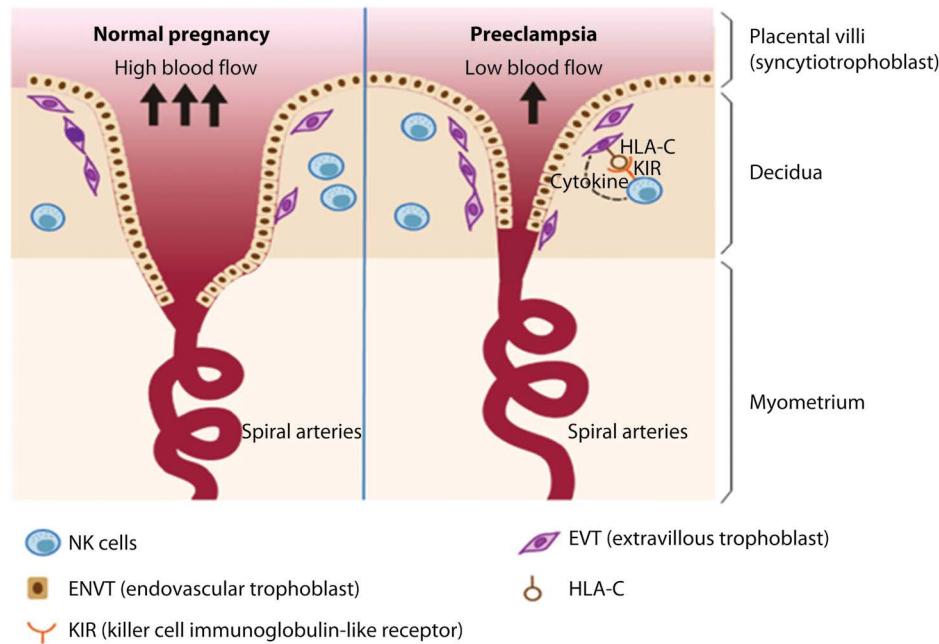


FIGURE 47.4 Schematic representation of normal (left picture) and poor placentation (right picture) during early pregnancy [53].

the main cause of the indication for oocyte donation, these disorders have also been observed in young recipients. This is what leads scientists to look for a cause other than age that can explain such situations, and the immunological aspect may be a possible answer to this unknown. In this way, as the donor's HLAs act as paternal, the presentation of foreign HLA ligands would be increased, making it easier for an immunological maladaptation to occur towards any of them [4].

In fact, one study observed that the rate of early miscarriage after DET with donated oocytes was higher in recipients with KIR AA genotype, followed by KIR AB, when comparing these groups with KIR BB patients. Similar results were recorded when measuring the LBR per cycle, being lower in KIR AA recipients, when they were compared with KIR AB and KIR BB patients [72]. A second study showed concordant results with the latter and stated that the proportion of pregnancies ending in loss is higher among KIR AA patients when a C2C2 euploid embryo is transferred than when C1C1 or C1C2 embryos are transferred [77].

According to these results, maternal-fetal HLA/KIR combinations would be notoriously influencing reproductive outcomes and immunologic selection of donors for KIR AA patients could be advised, selecting HLA-C1 among oocyte and/or sperm donors for patients who undergo oocyte donation and who express inhibitory KIR haplotypes [4]. This intervention would be inexpensive and low risk but confirmation of this association between reproductive disorders and certain KIR/HLA combinations is still required. Randomized controlled trials with adequate patient size are needed before implementing this selection in routine assisted reproduction protocols [69].

A research conducted in the Japanese population claims to find no relationship between the incidence of pre-eclampsia and the HLA/KIR genotype [78]. However, it was subsequently pointed out that the small sample size of this study did not allow conclusions supported by sufficient statistical power. Despite being, therefore, statistically flawed, it highlights the importance of considering ethnic differences when studying HLA/KIR combinations, as the KIR and HLA regions have evolved rapidly with the frequencies of KIR A haplotypes and HLA-C alleles carrying the C2 epitope varying among populations with a significant number of HLA/KIR combinations [69].

For example, Japanese show an increased frequency of the KIR A haplotype but, conversely, a low frequency of HLA-C alleles carrying the C2 epitope when compared to other populations, such as European [54]. It has been found, according to these population studies, that reproduction exerts an important selective pressure for KIR and HLA-C diversity, so, it is likely that the regions encoding these genes have evolved rapidly to prevent risky pregnancy combinations. This would represent further evidence of the relationship between KIR/HLA genotype and reproductive outcomes [7, 69].

Finally, it is important to note that, although studies on the deleterious effect of the KIR AA genotype are the most numerous, it is not only the presence of inhibitory KIR that has been associated with adverse reproductive effects. Thus, an overactivation of uNK cells, which could occur in patients carrying the KIR BB genotype (especially if KIR2DS1 is present) would also affect reproductive outcomes. In fact, a greater frequency of KIR2DS1 has been correlated with higher birth weight [74].

It has been proposed that this exaggerated activation could lead to enhanced production of angiogenic factors and cytokines. This, in turn, would lead to increased angiogenesis and blood supply. The elevated arrival of oxygen to the maternal-fetal interface

is what could affect adequate placentation, as it is known that trophoblastic invasion is favoured under conditions of hypoxia [7]. Placental tissue is quite sensitive to the concentration of oxygen during the first trimester of gestation, due to the low presence of antioxidant enzymes [79]. Therefore, conditions of elevated partial pressure of oxygen, together with the possible high oxidative stress that this would bring, can be detrimental for a correct invasion of the trophoblast and, thus, for the achievement of a healthy pregnancy.

Corroborating this theory are articles that have reported increased production of angiogenic factors in women with RM [80]. Likewise, an increased number of blood micro-vessels have been observed in the endometrium of these women, demonstrating elevated angiogenesis [81].

This explanation could also provide an answer to the elevated numbers of uNK cells found in some patients with RM. An increase in this cell type would lead to the same effect described for its overactivation. Similarly, increased angiogenesis has been associated with higher progesterone levels in patients. This hormonal imbalance would affect the expression of certain genes of the killer cell lectin-like receptors (KLRs) family, also involved in the synthesis of angiogenic factors [82].

Taking all this into account, future research on uNK cell population, their functions and the impact that their KIR receptors together with HLA ligands may have on pregnancy could open the door to new approaches to predict and prevent pregnancy disorders.

Immunomodulation treatments: Fighting against the enemy?

As seen so far, regulated trophoblastic invasion is absolutely necessary for the well-being of both the mother and the embryo. Despite this invasive process, no phenomena of immune rejection by uNK cells have been observed. Moreover, it has been established that uNK cells act positively in favouring the invasion of maternal tissue by EVT cells and the proper construction of the placenta [52].

However, the concept idea that uNK cells, because of their supposed cytotoxic activity, could attack trophoblastic cells leads many physicians to offer blood tests to measure the number of such cells and even immunomodulation treatments to the patients, associating an increased quantity of these lymphocytes with poorer results in IVF treatments [7]. These supposed therapies include IVIG, intralipids, anti-TNF- α , paternal leukocyte immunization, steroids, progesterone, and G-CSF.

IVIG, intralipids, anti-TNF- α have already been discussed in the section on T cells, as they have been used simultaneously both to modulate the Th1/Th2 balance and to try to reduce the cytotoxicity and inflammation presumably associated with increased levels of NK cells. Indeed, current studies do not show changes in reproductive outcomes sufficiently robust to support the routine introduction of these treatments [23].

Another alleged immunomodulatory therapy to be considered is lymphocyte immunotherapy (LIT), which began to be offered in the 1980s and was based on injecting women with purified lymphocytes from their male partners [83]. The scientific rationale for this practice was to avoid unwanted immune responses that could harm the pregnancy by prior exposure to paternal antigens.

Until the FDA finally banned its use in 2002, this so-called therapy continued to be offered to patients, especially to those who, after a blood test, showed high levels of NK cells, supposedly capable of attacking the fetus. All studies carried out on

lymphocyte injection concluded that such treatment was unable to improve reproductive outcomes and, therefore, its use in the clinical setting should not be considered [7, 84].

Some studies report positive results with the use of corticosteroids for patients who suffer recurrent miscarriages. The use of these drugs is based on their anti-inflammatory and immunosuppressive capabilities. Thus, they would be expected to alter the immune environment, decreasing cytotoxicity and hyperactivity, proposed causes of RM. For example, taking prednisolone seems to favour the group of patients suffering this reproductive disorder, whereas no differences in terms of gestation rate are observed for the rest of the patients undergoing ICSI [85].

These statements do not correspond with the results obtained later by other investigators, who treated patients with reproductive failure with prednisolone and found no difference in pregnancy outcomes, but a decrease in uNK cells in the corticosteroid group [86]. Thus, they assumed that such drugs would act by reducing total uNK cells, but such an effect is not reflected subsequently in the reproductive outcomes in this group of patients.

In this regard, it is worth mentioning another study in which a combined treatment of aspirin, doxycycline, and prednisolone was used for unselected patients [87]. This study did not show any difference in reproductive outcomes between the treated and control groups when performing fresh embryo transfers. Interestingly, for frozen embryo transfers, there was a decrease in the live birth rate in the treatment group.

These results should be considered when administering medication to patients, as it could even negatively impact their reproductive outcomes by prescribing corticosteroids indiscriminately, without patient selection or sufficient research to support the use of these therapies. Numerous criticisms have arisen in relation to the studies carried out to determine the possible benefits, not only of the use of corticosteroids but of all the immunomodulatory therapies proposed in ART. In this regard, the heterogeneity of investigations stands out, there being no standardization in terms of patient groups, form of administration, time of use, or dosage employed, which prevents comparison among studies [7, 84].

No cut-off point has yet been established to determine which values of pbNK and uNK cells should be considered out of normality. Each article uses its own percentages to make this division, which, again, increases variability and prevents reproducibility of these investigational therapies. Also, only supposedly elevated cellular values are usually taken into account, without paying attention to cases that might be below normal. In this way, the proposed therapies could act by further decreasing NK cells in these patients, causing possible detrimental effects [7].

A consensus on these cut-off levels and the NK cell measurement protocols to be used is therefore required before this analysis can even be considered as a possible diagnostic tool. Likewise, as explained earlier, it is essential to establish a distinction between uNK and pbNK cell populations, as they have different functions, phenotypes, and localization, and the measurement of one has not yet been correlated with the other [40, 84]. Therefore, searching for markers in peripheral blood rather than directly in the endometrium would not be an adequate approach to study reproductive aspects [4].

Although it seems that certain therapies could favour the outcome of specific patient groups, subsequent reviews of the most used immunomodulatory treatments in ART (IVIG, LIT, G-CSF ...) determine that there is not enough evidence to show that such medical practices are able to prevent RM or to improve

the reproductive outcome of patients, even in selected groups, with high NK cell numbers/activity [88, 89]. The heterogeneity of the trials conducted, and the paucity of high-quality scientific evidence oblige to keep these treatments in the context of research. Many studies are still required before their translation, if this occurs, to the clinical setting.

For these reasons, it has been continuously discouraged to offer these treatments as routine for ART patients. The use of immune agents as empirical is not harmless: IVIG during pregnancy has been associated with certain side effects, including headache, skin rash, predisposition to thrombosis, or anaphylactic shock. There also seems to be a link between corticosteroid use during early pregnancy and a relatively higher risk of orofacial cleft palate; and treatment with LIT has resulted in significant maternal complications, such as hepatitis, cytomegalovirus, flu-like symptoms, transfusion reactions, or autoimmune problems [35, 84].

The introduction of such therapies should be carried out with caution, and treatment should be personalized according to the immunological situation of each patient. Well-designed trials with a larger number of subjects could help to identify and treat patients who may benefit from these therapies in the future. Until now, individuals have been treated indiscriminately, sometimes using the RM as the sole specification, since some publications had associated this and others undesirable reproductive conditions with increased levels of NK cells [48, 49].

However, other articles failed to find this correlation and some meta-analyses and systematic reviews emerged to dispel the doubts regarding this subject; stating that further research and more uniform criteria are needed before NK cell assessment can be recommended as a diagnostic tool [50, 51]. It is claimed, therefore, that there is not any biological reason to reduce the maternal immune system to a simplistic analysis, the NK cells tests, and use these analyses in routine clinical practice as diagnosis of immune factor, much less the immunotherapy treatments based on them (see Table 47.1).

Much remains to be elucidated about the role of the immune system in reproduction, and it is important to be sure, before routine use, how these immunomodulators affect the activity of uNK cells and their interactions, which have been shown to be so essential during pregnancy. The roles played by uNK cells, angiogenesis, and EVT invasion are central processes of reproduction, and any aspect that could alter them must be profoundly investigated. The immune system at maternal–fetal interface has a huge complexity. All these interactions, not only one subset of cells (NK cells), have to be considered in those patients where immune factor is suspected, in order to propose an individualized therapeutic approach.

Autoimmunity: Reproductive consequences

Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by arterial, venous, or small vessel thrombosis, thrombocytopenia, and pregnancy morbidity (miscarriages, stillbirth, and severe pre-eclampsia). These episodes are associated with the presence of antiphospholipid antibodies (APAs), the most common being represented by anticardiolipin antibodies (aCL), anti-beta 2 glycoprotein-I (a β 2GPI), and lupus anticoagulant (LAC) [90, 91].

The current recommended criteria to diagnose this autoimmune disorder were established by Sapporo, many years ago, in 2006 [92]. They include the presence of one clinical criterion

TABLE 47.1 Summary of Immunomodulator Studies in ART

Immune therapy	Type of study	Study description	Results	Publication	Use in ART
TNF-α blockers	Retrospective observational study	76 women undergoing fresh IVF/ICSI cycles and with TNF- α /IL-10 cytokine elevation were treated with both Adalimumab and IVIG. Their reproductive results were compared to baseline IVF characteristics and patient history	Only implantation rate was statistically significant when comparing between women with least optimal cytokine conditions and those with the most optimal cytokine	[24]	Despite some favourable publications, no usefulness in clinical setting has been proved. Safety concerns about its use during pregnancy.
	Prospective cohort study	75 sub-fertile women with Th1/Th2 cytokine elevation were divided into four groups: patients using both IVIG and Adalimumab, patients using IVIG, patients using Adalimumab and patients using no IVIG or Adalimumab	Significant improvement in implantation, clinical pregnancy and live birth rates for group I versus group IV and for group II versus group IV	[25]	
Intralipids	Randomized placebo-controlled double-blinded trial	296 women were enrolled and randomly assigned to receive intralipid or saline infusion on the day of oocyte retrieval.	Intralipid supplementation did not increase frequency of chemical pregnancy	[29]	This therapy does not improve clinical outcomes in any case. Its implementation is unnecessary and inadvisable
	Prospective cohort study	Women aged 40-42 with a previous history of miscarriage or who failed to conceive despite previous embryo transfer who entered an IVF program were offered intravenous intralipid therapy	No clinical pregnancies in those receiving intralipid vs. a 40% clinical and a 30% live delivered pregnancy rate in the untreated controls	[30]	
IVIG	Retrospective observational study	76 women undergoing fresh IVF/ICSI cycles and with TNF- α /IL-10 cytokine elevation were treated with both Adalimumab and IVIG. Their reproductive results were compared to baseline IVF characteristics and patient history.	Only implantation rate was statistically significant when comparing between women with least optimal cytokine conditions and those with the most optimal cytokine	[24]	No clinical usefulness has been demonstrated to support its implementation. Its use has been linked to side-effects.
	Systematic review and meta-analysis	Investigation of the effects of IVIG on implantation rate, clinical pregnancy rate, live birth rate, miscarriage rate, and live birth rate per embryo transferred. The PubMed, EMBASE, and CNKI databases were searched up to June of 2013 and 10 studies were included.	Higher implantation rate, pregnancy rate and lesser miscarriage rate in the group treated with IVIG. These positive results were not subsequently reflected in the live birth rate.	[31]	
	Randomized comparative study versus placebo	39 women with history of abortions or implantation failure were enrolled. 18 patients were randomized in the IVIG treatment group (group A) and 21 in the placebo arm (group B)	Higher implantation rate in the group treated with IVIG but, subsequently, no difference in pregnancy rate was registered.	[32]	

(Continued)

TABLE 47.1 Summary of Immunomodulator Studies in ART (Continued)

Immune therapy	Type of study	Study description	Results	Publication	Use in ART
IVIG	Case-control study	330 patients were enrolled and treated with IVIG Inclusion criteria was age 18-49, with ≥ 2 failed IVF/ oocyte donation with at least 2 good quality embryos transferred per cycle. 2 control were chosen for each case.	No significant differences were registered between case and control for any of the reproductive outcome, except for miscarriage rate, which was higher in women treated with IVIG and undergoing IVF.	[33]	No clinical usefulness has been demonstrated to support its implementation. Its use has been linked to side-effects.
	Systematic review	Embase, LILACS, MEDLINE, PsycINFO, CENTRAL and CINAHL databases from 1946 to present were searched and three studies evaluating the use of adjuvant therapies in women undergoing ART with elevated NK cell numbers and/or activity were included	Some data showed that adjuvant therapies (mainly IVIG) in this selected population seem to confer some benefit on ART outcome, but the evidence is scarce and of poor quality	[34]	
LIT	Double blind trial	22 women were injected with the husband's lymphocytes and 27 with their own lymphocytes.	The pregnancy outcomes were significantly better in the immunized group than the control	[83]	No proven usefulness in clinical setting and safety concerns for most patients.
	Systematic review	Cochrane Pregnancy and Childbirth Group's Trial Register (11 February 2014) were searched and 20 randomized trials of immunotherapies to treat women with 3 or more prior miscarriages were included	No significant differences between treatment and control group in term of subsequent live birth	[84]	
	Meta-analysis	Relevant publications were searched from databases and the included randomized controlled trials (RCTs) investigated effects of prednisolone administration in women with unexplained RM or during ART	Prednisolone therapy improves pregnancy outcomes in women with idiopathic RM, in terms of live birth, successful pregnancy and miscarriage rate.	[85]	This immunomodulator does not seem to improve reproductive outcomes in general population. Conversely, some negative results have been registered.
Corticosteroids	Retrospective cohort study	136 women diagnosed with RM or RIF were included and those with high numbers of uNK (N = 45) were treated with prednisolone for one month. Pregnancy outcomes and complications were compared with those who did not receive this corticosteroid.	There was no difference in any of the pregnancy outcomes or complications between women who had received prednisolone and those who had not.	[86]	
	Matched case-control study	485 women (cases) received a combined co-treatment with aspirin, doxycycline, prednisolone. Reproductive outcomes were compared with those of 485 women who were not treated and constituted the control group.	No significant differences were found in fresh cycles between cases and controls for the pregnancy outcomes analysed. With frozen embryos, the LBR was lower in the treatment group.	[87]	

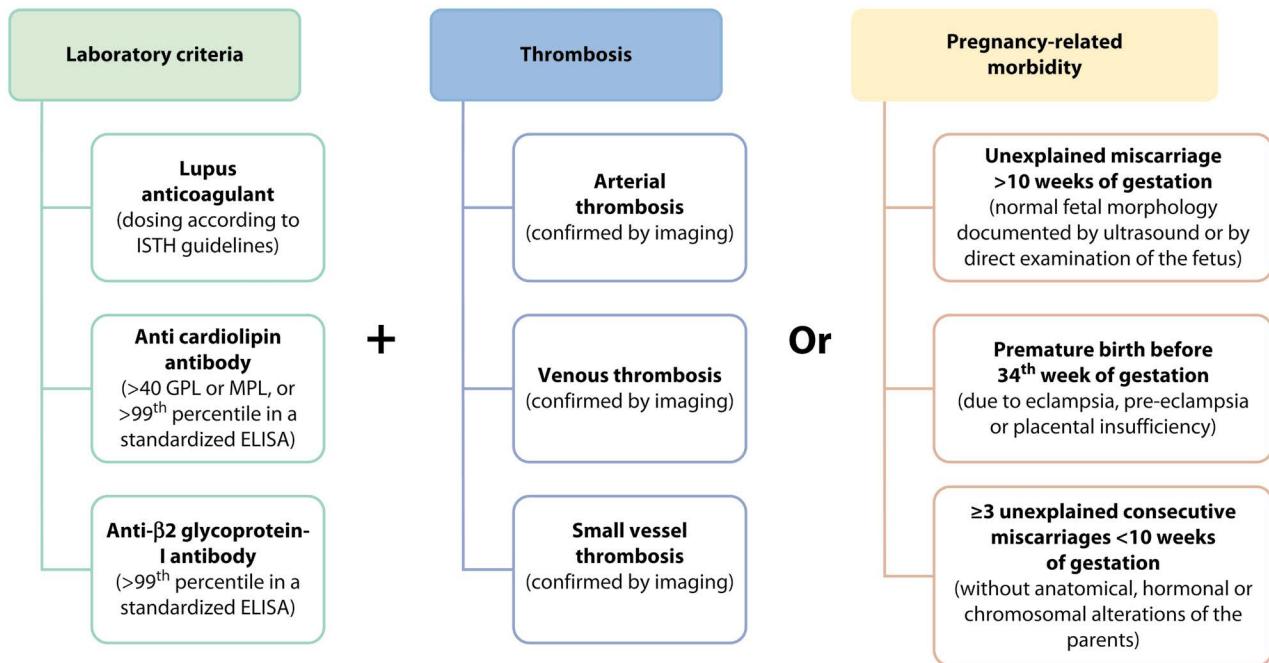


FIGURE 47.5 Summary of criteria for antiphospholipid syndrome (APS) diagnosis according to Sapporo criteria. Abbreviations: ISTH: International Society of Thrombosis and Haemostasis. GPL: Glycopeptidolipid; MPL: Monophosphoryl lipid A [90].

(thrombosis or pregnancy-related morbidity) and positive values of at least one of the three APAs, in two determinations taken 12 weeks apart (Figure 47.5).

Even though the diagnosis criteria published 16 years ago mentioned the need of three unexplained consecutive miscarriages, the current recommendations reduced the number of miscarriages at two and no need to be consecutive.

The relationship between female reproductive disorders and APS is based, firstly, on the binding of these APAs to trophoblastic cells, causing abnormal trophoblastic endovascular invasion. Likewise, the alteration of VEGF levels, common in this syndrome, would lead to problems in the formation of the blood vessels required for gestation [3]. The significant decrease in maternal blood flow to the placenta, causing ischemic injury, lack of nutrients to the fetus, and increased oxidative stress, can damage the placenta and leads to restricted intrauterine growth, pre-eclampsia, and fetal loss, among other undesirable outcomes.

In addition, it has been established that these antibodies can affect endometrial decidualization, thus compromising implantation; while favouring a proinflammatory state in the endometrium, which also interferes with the achievement of a healthy gestation [93].

However, there is some controversy regarding the involvement of APAs in certain disorders, such as recurrent implantation failure (RIF) [3]. Research on this subject is mostly case-control studies or small cohorts; poor data to demonstrate the impact of these antibodies on RIF.

APS is usually treated in the context of ART with aspirin and heparin. The former prevents platelet aggregation through its antithromboxane effects and may counteract APA-mediated hypercoagulability in the decidual space. On the other hand, heparin, in addition to its known anticoagulant effects, may help patients suffering from this syndrome by preventing the binding of APAs to trophoblastic cells [3].

Studies conducted to determine the efficacy of these treatments conclude that their use in unselected IVF or ICSI patients does not improve reproductive outcomes and that the evidence for implementing them in routine clinical procedures is weak. However, this situation is different if patients have antiphospholipid syndrome. In fact, prophylactic prescription of heparin and low-dose aspirin is recommended for women with APAs who have suffered recurrent miscarriages (two or more), or obstetric complications such as pre-eclampsia, stillbirth, FGR, and preterm birth [91]. Other studies, however, suggest that testing for these autoantibodies is not recommended in the initial assessment of infertility, and is only to be considered when iterative episodes of gestational losses have already occurred, at which point their use would become unquestionable [94], but may be too late for ART patients.

To conclude, the relationship of APAs with negative reproductive outcomes and the benefit of the treatments for those patients are shared by most authors.

Other autoimmune situations and their impact on reproduction

Although APAs are the autoantibodies most commonly associated with pregnancy disorders, there are others that also appear to interfere with the achievement and/or progression of pregnancy. An example of this is antinuclear antibodies (ANAs), which are non-specific antibodies targeting components of the cell nucleus [95].

Their connection with reproductive problems is evidenced in several publications, including a study that observed an increased prevalence of such antibodies in infertile women versus a fertile female population [96]. Other researchers have also observed an association between the presence of these ANAs and a lower pregnancy and implantation rate [94].

The mechanism by which these autoantibodies affect reproduction is still unknown, with some authors proposing a possible

deleterious effect on oocytes and embryos [94]. Therapies considered in the context of ART for these patients have been the use of glucocorticoids and low-dose aspirin. However, these are only suggestions in the research setting and are not yet part of the clinical context.

With the studies carried out, the connection between the presence of ANAs in the blood and certain disorders such as RM seems to be a reality, although further investigations are needed to finally confirm this link. The continuation of research in this area will probably allow identifying a possible role for ANAs in the identification of a subset of women eligible for various forms of immunotherapy [95].

Similar to the case of ANAs, autoantibodies targeted against different parts of the ovary (anti-ovarian antibodies, AOAs) have been studied for years in the context of reproductive autoimmunity. Some of the possible antigenic targets described for these antibodies are the β -subunit of follicle stimulating hormone (FSH), the corpus luteum, the zona pellucida, and granulosa cells. In this regard, several studies have correlated the presence of AOAs with certain conditions such as premature ovarian insufficiency (POI). Nevertheless, their role in the development of this and other pathologies is unknown. In addition, the low specificity of the existing tests for these autoantibodies can lead to a high rate of false positives and to a lack of validation of the results. These aspects greatly limit their usefulness as potential biomarkers and prevent them from being considered as a routine analysis when studying autoimmunity in patients [97].

Another type of autoantibody also related to reproductive function is antithyroid antibodies. There is high-quality evidence that the presence of thyroid peroxidase (TPO) antibodies, the most common in this type of autoimmunity, is strongly associated with miscarriage, pre-term birth, and the development of thyroid disease in pregnancy. Likewise, evidence has been found that it may also be connected to a higher risk of placental abruption, premature rupture of membranes, and maternal anaemia [98].

However, these results are debatable when only ART patients are included in the investigations. There are studies that reiterate the previous conclusions, showing an association between the presence of antithyroid antibodies and lower fertilization, implantation, and pregnancy rates [99]. On the other hand, a subsequent meta-analysis examined the effect of antithyroid antibodies in euthyroid ART patients (defined as those with normal triiodothyronine (T3) and thyroxine (T4) concentrations and no history of thyroid disease) and reached conclusions that differed from those shown by the studies described so far [100]. These researchers established that, indeed, patients with antithyroid antibodies showed worse reproductive outcomes in cycles; but this was not always the case. The measurement of thyroid stimulating hormone (TSH) made it possible to constitute a group of women who were known not to have subclinical hypothyroidism. Interestingly, in this group no differences in reproductive outcomes were found between those with and without antithyroid antibodies.

A recent review and meta-analysis [101] has tried to answer some of the controversies regarding the association between reproductive success and the presence of antithyroid antibodies. This research studies the administration of levothyroxine to women with subclinical hypothyroidism and its relationship with pregnancy outcomes, as well as the association of thyroid autoimmunity to recurrent pregnancy loss.

The results obtained suggest that the administration of levothyroxine does not benefit euthyroid women and establish an

association between thyroid autoimmunity (TAI) and RPL. Although it is known that thyroid hormones are essential in oocyte maturation and the menstrual cycle, the exact mechanism by which antithyroid antibodies may be affecting the development of pregnancy is incompletely elucidated, as this phenomenon can be observed even in the absence of thyroid dysfunction [94].

Some studies have pointed out a relationship between antiphospholipid syndrome [102] and thyroid autoimmunity, suggesting common pathophysiologic processes and genetic background. In fact, the prevalence of anticardiolipin antibodies or lupus anticoagulant is significantly higher in women with TAI when compared to women without TAI.

Treatment for immune dysfunctions induced by APAs does not include levothyroxine or intravenous immunoglobulin (IVIG). That is why studies based on treating euthyroid women with RPL and thyroid autoimmunity with levothyroxine failed to show any positive impact on their pregnancy outcomes.

The evaluation of women with RPL should include APAs screening in line with current recurrent miscarriage guidelines, paying special attention to women with thyroid autoimmunity and euploid embryo losses. More studies involving a careful selection of patients, euploid embryo transfers, and autoimmune, molecular, and transcriptomic analysis of molecules involved in placentation are needed to identify whether the presence of thyroid autoimmunity is a marker of an immune imbalance which could affect maternal-fetal tolerance and increase the risk of RPL.

Routine screening for antithyroid antibodies is not advocated at present but should be considered for "high-risk" populations such as women with a history of recurrent miscarriage or preterm birth [98]. It is essential to explore causal pathways linking these antibodies and adverse outcomes and subsequently develop new treatment strategies to improve pregnancy success. Likewise, thyroid supplementation is recommended in those cases of overt and subclinical hypothyroidism.

All these autoantibodies (APAs, ANAs, TPO etc.) are a signal of hyperactivity, which, by itself, has already been related to worse reproductive outcomes. Moreover, not only autoantibodies but autoimmune diseases in general (rheumatoid arthritis, systemic lupus erythematosus, antiphospholipid antibody syndrome, systemic sclerosis), which predominantly affect women during their reproductive years, are associated with pregnancy disorders, as they represent the best-known cause of hyperactivity and proinflammatory states [103].

Some examples of certain autoimmune diseases that should be highlighted in the context of infertility are diabetes and celiac disease (CD). Firstly, diabetes is a complex disease classically classified into Type 1 and Type 2, division that does not include all metabolic disorders related with impaired insulin secretion or action. Type 1 diabetes is an autoimmune disease characterized by immunological pancreatic attack by autoreactive T cells and autoantibodies with severe loss of insulin secretion. Around 5%–14% of patients classified with Type 2 diabetes have diabetes-associated autoantibodies.

The term latent autoimmune diabetes in adults (LADA) has been introduced for this autoimmune diabetes characterized by adult onset, presence of diabetes-associated autoantibodies, and more frequent need for insulin treatment than patients with classical Type 2 diabetes. LADA is the most prevalent form of adult-onset autoimmune diabetes and probably the most prevalent form of autoimmune diabetes in general.

In the reproductive field, most of the tests to detect functional glucose impairment are used during pregnancy and less is known about its usefulness in the preconception period, even more so in ART. A recent study [104] observed a significantly increased live birth rate (LBR) per cycle after a precise diagnosis and adequate metabolic status compared with LBR/cycle without pancreatic autoimmunity control.

The diabetes-associated autoantibodies (DAA) appear even years before LADA diagnosis. The current preconceptional protocols do not include tests to detect pancreatic autoimmunity, and affected women presenting RIF or RM are often misdiagnosed. Immune or metabolic routine screening for all infertile couples is not advised, but a tailored approach is useful for some subsets of patients having "silent" immune or metabolic disorders.

For glycaemic disorders, only fasting glucose is tested before starting ART and this determination falls short in some subsets of patients. We reported [104] that patients with RM or RIF of unknown aetiology diagnosed with thyroid autoimmune disorders, family history of diabetes, and impaired insulin response after oral glucose tolerance test (OGTT) could be considered as a subset of patients, candidates for further specific autoimmune tests to rule out DAA.

In conclusion, it is quite easy, by clinical and metabolic characteristics described, to identify patients with pancreatic autoimmunity or LADA and recommend a correct treatment with a positive impact in their preconception management and reproductive result after ET.

On the other hand, celiac disease (CD) is nowadays recognized as an immune-mediated systemic disease related to dietary gluten ingestion in genetically susceptible children and adults. Undiagnosed CD can be associated to recurrent spontaneous abortions, intrauterine growth restriction, low birth weight, delayed menarche, early menopause.

Including infertility in the group of CD-associated conditions caused a big controversy, and a consensus has not been reached due to the contradictory results found in the literature. Despite the increasing number of papers relating CD and adverse pregnancy outcomes, there is not unanimous consensus about considering women with reproductive problems as a risk group for CD. We reported recently better reproductive outcomes in celiac patients under a gluten free diet (GFD) compared to a normal diet [105].

Current knowledge does not allow giving specific recommendations about general screening of CD in women with recurrent reproductive failure. Further studies are still needed, preferentially with a prospective design and careful handling of the beneficial effect of the GFD on reproductive outcomes.

The proven relationship between autoimmunity and poor reproductive outcomes makes it necessary to personalize, in some way, ART treatments for these patients. Preconception counselling, strict disease control, and embryo transfer planning based on the clinical stability of the autoimmune disease are the essential steps to follow in the management of autoimmunity, to maximize reproductive options, as well as avoid autoimmune disorders in the new-born [106].

It is, therefore, adequate to perform blood tests in patients with manifested hyperactivity to demonstrate its autoimmune cause, if autoantibodies are present (e.g. antiphospholipid syndrome) and, possibly, relate it to an autoimmune disease susceptible of being treated. The associations found between these situations of autoimmunity and reproductive disorders demonstrate, once

again, the importance of immunology in reproduction, having to take it into account when carrying out ART, especially in patients with unknown causes of infertility and not immunologically tested.

Local infections and pregnancy

Beyond the presented role in establishing adequate fetal tolerance and participating in various processes of early placentation, the immune machinery located in the endometrium is also involved in the defence against pathogens. Macrophages and dendritic cells are the main immune components responsible for this, increasing their number and activity in the presence of local pathogens [107].

With their high phagocytic capacity, they clear infected, apoptotic cells and cell debris. In macrophages, this process is precipitated by their Toll Like Receptor 4 (TLR4), which is able to recognize lipopolysaccharides (LPS), the main components of the cell surface of gram-negative bacteria [108]. Macrophage activation ends with Treg recruitment and immunoregulation to re-establish endometrial tissue homeostasis.

There are, however, circumstances of persistent inflammation, in which such homeostasis is lost. It is then that we speak of chronic endometritis (CE). This situation is caused by recurrent infections of certain microorganisms, including *Escherichia coli*, *Streptococcus spp*, *Staphylococcus spp*, *Chlamydia*, *Mycoplasma*, *Ureaplasma*, yeast, and some viruses. Pelvic pain, dysfunctional uterine bleeding, dyspareunia, and increased leucorrhoea are among the symptoms of this condition [20].

CE has been associated with poorer reproductive outcomes, with this condition being frequently observed in cases of RM and RIF. Antibiotic treatment prescribed to combat the causative microorganisms appears to increase reproductive success and equalize it to that of women without CE. Some authors have hypothesized that the underlying cause of such fertility impairment could be a local immune imbalance, which would be altering some aspects such as endometrial receptivity. Supporting this idea, an increase in CD68+ macrophages has been reported in cases of CE versus healthy patients [20].

However, a similar increase in Treg cells has been observed in these patients, which would correspond to a protective immune response facing this proinflammatory environment. Therefore, and because more evidence would be needed in this regard, it cannot be determined that it is an immunological dysregulation at the endometrial level that leads to these reproductive consequences [108].

Nevertheless, CE diagnosis and subsequent treatment is of vital importance in ART patients, thus favouring their chances of success. In addition, infections should be highly monitored by healthcare professionals, not only during reproductive treatment but throughout the pregnancy and subsequent delivery.

In this respect, several authors agree that pregnant women are especially susceptible to viruses, being at increased risk for severe illness and mortality. Moreover, the consequences of such infections would not only affect maternal health but could also extend to the child, through viral transmission during pregnancy or childbirth.

Among the viruses of most concern and most present in episodes of intrauterine infections, cytomegalovirus (CMV) stands out, showing a seroprevalence of 86% in women of childbearing age. This is an enveloped double stranded DNA herpes virus, and similarly to other viruses of the same family, it becomes latent

after a primary infection but can reactivate with renewed viral shedding or from a new strain [109].

It has been established that infection with CMV during the first weeks of gestation may lead to miscarriage, through mechanisms that affect immunological maternal–fetal crosstalk. In this sense, the presence of CMV in EVT cells may modulate the activity of uNK cells, towards increased cytotoxic properties. This favours apoptosis in the uterine micro-environment, while maternal–fetal tolerance is compromised [110].

In addition to affecting the continuation of pregnancy, maternal primary CMV infection can cause fetal infection in 30% of cases during the first trimester of gestation, increasing this percentage with gestational age [111]. However, most congenital infections are asymptomatic at birth (75%–90%), with fewer possible consequences for the child the later the transmission during pregnancy.

It should be noted, though, that approximately one-quarter of these asymptomatic births will develop symptoms in the first two years of life. The main CMV-related sequelae are sensorineural hearing loss (SNHL), neuro-disability, and cerebral palsy. Prevention during pregnancy, the use of CMV hyperimmune globulin to avoid symptomatic congenital infection in high-risk pregnancies of infected women, and a close follow-up are currently the approaches to face CMV [109].

Another virus of great concern lately, due to its recent appearance, is Covid-19. Its consequences for reproduction and pregnancy, as well as the existence of vertical transmission, are being studied and much remains to be described.

According to a recent meta-analysis, the majority of children born to Covid-positive mothers who acquire this infection do so through postpartum transmission. However, some cases of intrauterine fetal mother-to-child transmission have also been reported [112].

Although many cases remain asymptomatic, a worrisome condition that is being associated with these Covid-positive pregnancies is the occurrence of chronic histiocytic intervillitis. This disorder consists of the accumulation of inflammatory mononuclear cells in the placental intervillous space and has been strongly associated with poor obstetric outcomes, which include miscarriage, fetal demise, intrauterine growth restriction, and preterm delivery [113].

Further investigations of more pregnancies will shed light on the issues raised by this viral infection, which concern both mother and child. Prevention is again the best tool to avoid these situations, considering that pregnant women are excluded from many of the studies on potential therapies in the fight against Covid-19 [114].

This, together with the controversies about the possible greater vulnerability of pregnant women to Covid-19, generates doubts and concerns in the population, which should be resolved as more and more research is conducted on this new pathogen.

Implications of local immunological imbalances in reproductive and metabolic disorders

Having presented the main immune components related to fertility (T cells, NK cells, autoantibodies ...), we consider it necessary to carry out a brief analysis of the local imbalances that have been described in various situations in which the initiation and/or adequate progression of pregnancy is affected. This disequilibrium

between pro- and anti-inflammatory states, already introduced, will now be reviewed by disease, and the reproductive alterations they cause will be described.

First of all, we will focus on adenomyosis and endometriosis, as two of the conditions that have been most often related to local immunological alterations. In the case of adenomyosis, the deviation towards local proinflammatory situations (with increased cytokines such as TNF- α , elevated Th17 lymphocytes, and decreased Treg cells), together with described dysfunctions of uNK cells, are related to lower implantation rates in these patients [17, 115]. These studies suggest that a local increase in the oestradiol level and/or a decrease in the progesterone level, along with the modulation of the immune system, may be the explanations for the poorer reproductive outcome observed in women affected by adenomyosis.

Similarly, a wide range of proinflammatory changes in the uterine immune profile have been described in patients affected by endometriosis. Macrophages, immature dendritic cells, and Treg cells behave differently. NK cells display abnormal activity in the endometrium of affected women and a Th17/Treg cell imbalance has also been observed. This proinflammatory state acts as a pathogenetic mechanism associated with implantation failure [116].

In addition, aberrant genome-wide signatures, caused by steroid hormones in patients suffering endometriosis have been detected, while a higher expression of inhibitory KIR has been related to this condition [117, 118].

Besides these reproductive diseases, immune imbalances towards proinflammatory states have also been observed in metabolic disorders, such as PCOS, insulin resistance syndrome, obesity, etc. In these situations, the process of decidualization, the differentiation of endometrial fibroblasts into decidual secretory cells, is usually altered.

This seems to be caused by the fact that hyperinsulinemia and insulin resistance impair the necessary signalling by progesterone for this process to take place, affecting endometrial receptivity [118]. It is well known that both conditions are present in obesity and PCOS, two common metabolic disorders associated with subfertility.

Moreover, women with PCOS have altered endometrial immune cells [22] that promote a state of chronic low-grade inflammation, which appears independently of obesity and is related to reproductive failure [119]. Clinical characteristics associated with PCOS may contribute to the dysregulation of the endometrial expression of sex hormone receptors and coreceptors; increase endometrial insulin resistance; and, as a result, favour chronic low-grade inflammation, immune dysfunction, abnormal endometrial gene expression or cellular abnormalities that have been commonly associated to recurrent reproductive failure.

Conclusions

This chapter has provided a review throughout the multiple components of the immune system that play a role in reproduction and may be considered in ART, enlightening the importance of some cells like macrophages, T and NK lymphocytes. However, the enormous number of elements (cells, autoantibodies, receptors ...) that can affect reproductive outcomes demonstrate its undeniable complexity. At the same time, the impossibility to consider each component in isolation, due to the specific interactions they establish, hinders research in this subject.

Immunomodulation treatments to correct lymphoid disbalances and to avoid inflammation conditions should be considered for those patients with a clear identified immune factor as an individualized protocol (AAS and heparine for APS, gluten free diet for celiac disease, metabolic control for LADA patients etc.). Besides, the impact of HLA/KIR combinations on reproductive outcomes may lead to innovative approaches as immunological donor selection.

On the other hand, the management and treatment of women with diagnosed endometrial infections or autoimmune diseases are crucial in order to maximize their reproductive success. Adequate control of the progression of these conditions is highly effective in these patients.

The reproductive aspects associated with immunological issues are numerous, and, while it is true that the achievement of pregnancy and, subsequently, of live birth is completely multifactorial, immunology highlights as an important area that must not be underestimated.

References

- Luke B. Pregnancy and birth outcomes in couples with infertility with and without assisted reproductive technology: With an emphasis on US population-based studies. *Am J Obstet Gynecol*. 2017;217(3):270–81.
- Yang Z, Liu J, Collins GS, et al. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: Results from a randomized pilot study. *Mol Cytogenet*. 2012;5(1):24.
- Fransasiak JM, Scott RT. Contribution of immunology to implantation failure of euploid embryos. *Fertil Steril*. 2017;107(6):1279–83.
- Alecsandru D, Garcia-Velasco JA. Why natural killer cells are not enough: A further understanding of killer immunoglobulin-like receptor and human leukocyte antigen. *Fertil Steril*. 2017;107(6):1273–8.
- Medawar P. Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. *Symp Soc Exp Biol*. 1953;7:320–38.
- Colucci F, Moffett A, Trowsdale J. Medawar and the immunological paradox of pregnancy: 60 years on. *Eur J Immunol*. 2014;44(7):1883–5.
- Diaz-Hernandez I, Alecsandru D, Garcia-Velasco JA, et al. Uterine natural killer cells: From foe to friend in reproduction. *Hum Reprod Update*. 2021;27(4):720–46.
- Moffett A, Chazara O, Colucci F. Maternal allo-recognition of the fetus. *Fertil Steril*. 2017;107(6):1269–72.
- Parham P. Antigen recognition by T lymphocytes. In: The Immune System, 4th ed. Garlans Sciences, eds. New York: Taylor & Francis Group, LLC, pp. 113–148, 2015.
- Makrigiannakis A, Karamouti M, Drakakis P, et al. Fetomaternal immunotolerance. *Am J Reprod Immunol*. 2008;60(6):482–96.
- Apps R, Gardner L, Moffett A. A critical look at HLA-g. *Trends Immunol*. 2008;29(7):313–21.
- Nancy P, Erlebacher A. T cell behavior at the maternal-fetal interface. *Int J Dev Biol*. 2014;58(2-4):189–98.
- Wegmann TG, Lin H, Guilbert L, et al. Bidirectional cytokine interactions in the maternal-fetal relationship: Is successful pregnancy a TH2 phenomenon? *Immunol Today*. 1993;14(7):353–6.
- Del Prete G, Maggi E, Romagnani S. Human Th1 and Th2 cells: Functional properties, mechanisms of regulation, and role in disease. *Lab Invest*. 1994;70(3):299–306.
- Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. *Immunol Today*. 1997;18(10):478–82.
- Tilburgs T, Roelen DL, van der Mast BJ, et al. Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory t cells from the peripheral blood to the decidua in human pregnancy. *J Immunol*. 2008;180(8):5737–45.
- Bourdon M, Santulli P, Jeljeli M, et al. Immunological changes associated with adenomyosis: A systematic review. *Hum Reprod Update*. 2021;27(1):108–29.
- Galgani M, Insabato L, Cali G, et al. Regulatory t cells, inflammation, and endoplasmic reticulum stress in women with defective endometrial receptivity. *Fertil Steril*. 2015;103(6):1579–86.e1.
- Wang WJ, Hao CF, Qu QL, et al. The deregulation of regulatory T cells on interleukin-17-producing T helper cells in patients with unexplained early recurrent miscarriage. *Hum Reprod*. 2010;25(10):2591–6.
- Li Y, Yu S, Huang C, et al. Evaluation of peripheral and uterine immune status of chronic endometritis in patients with recurrent reproductive failure. *Fertil Steril*. 2020;113(1):187–96.e1.
- Catalano PM, Shankar K. Obesity and pregnancy: Mechanisms of short term and long term adverse consequences for mother and child. *BMJ*. 2017;356:j1.
- Liu S, Hong L, Mo M, et al. Evaluation of endometrial immune status of polycystic ovary syndrome. *J Reprod Immunol*. 2021;144:103282.
- Hviid MM, Macklon N. Immune modulation treatments—where is the evidence? *Fertil Steril*. 2017;107(6):1284–93.
- Winger EE, Reed JL, Ashoush S, et al. Degree of TNF-alpha/IL-10 cytokine elevation correlates with IVF success rates in women undergoing treatment with adalimumab (Humira) and IVIG. *Am J Reprod Immunol*. 2011;65(6):610–8.
- Winger EE, Reed JL, Ashoush S, et al. Treatment with adalimumab (Humira) and intravenous immunoglobulin improves pregnancy rates in women undergoing IVF. *Am J Reprod Immunol*. 2009;61(2):113–20.
- Pottinger E, Woolf RT, Exton LS, et al. Exposure to biological therapies during conception and pregnancy: A systematic review. *Br J Dermatol*. 2017;178(1):95–102.
- Johansen CB, Jimenez-Solem E, Haerskjold A, et al. The use and safety of TNF inhibitors during pregnancy in women with psoriasis: A review. *Int J Mol Sci*. 2018;19(5):1349.
- Chambers CD, Johnson DL. Emerging data on the use of anti-tumor necrosis factor-alpha medications in pregnancy. *Birth Defects Res A Clin Mol Teratol*. 2012;94(8):607–11.
- Dakhly DM, Bayoumi YA, Sharkawy M, et al. Intralipid supplementation in women with recurrent spontaneous abortion and elevated levels of natural killer cells. *Int J Gynaecol Obstet*. 2016;135(3):324–7.
- Check JH, Check DL. Intravenous intralipid therapy is not beneficial in having a live delivery in women aged 40–42 years with a previous history of miscarriage or failure to conceive despite embryo transfer undergoing in vitro fertilization-embryo transfer. *Clin Exp Obstet Gynecol*. 2016;43(1):14–5.
- Li J, Chen Y, Liu C, et al. Intravenous immunoglobulin treatment for repeated IVF/ICSI failure and unexplained infertility: A systematic review and a meta-analysis. *Am J Reprod Immunol*. 2013;70(6):434–47.
- De Placido G, Zullo F, Mollo A, et al. Intravenous immunoglobulin (IVIG) in the prevention of implantation failures. *Ann N Y Acad Sci*. 1994;734:232–4.
- Zapata A, Pacheco A, Alecsandru D, et al. Does IVIG improve cycle outcome in women undergoing IVF/oocyte donation after failed cycles? *Fertil Steril*. 2013;100(3):S376.
- Polanski LT, Barbosa MAP, Martins WP, et al. Interventions to improve reproductive outcomes in women with elevated natural killer cells undergoing assisted reproduction techniques: A systematic review of literature. *Hum Reprod*. 2013;29(1):65–75.
- Duhem C, Dicato MA, Ries F. Side-Effects of intravenous immune globulins. *Clin Exp Immunol*. 1994;97(Suppl 1):79–83.
- Human Fertilization and Embryology Association. Immunological tests and treatments for fertility. Human Fertilization and Embryology Authority, 2021. Accessed January 21, 2022. Available from: <https://www.hfea.gov.uk/treatments/treatment-add-ons/immunological-tests-and-treatments-for-fertility/>.

37. Versteeg R. NK cells and T cells: Mirror images? *Immunol Today*. 1992;13(7):244–7.
38. Trundley A, Moffett A. Human uterine leukocytes and pregnancy. *Tissue Antigens*. 2004;63(1):1–12.
39. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol*. 2001;22(11):633–40.
40. Moffett A, Shreeve N. First do no harm: Uterine natural killer (NK) cells in assisted reproduction. *Hum Reprod*. 2015;30(7):1519–25.
41. Pantazi A, Tzonis P, Perros G, et al. Comparative analysis of peripheral natural killer cells in the two phases of the ovarian cycle. *Am J Reprod Immunol*. 2010;63(1):46–53.
42. Kammerer U, Rieger L, Kapp M, et al. Immunocompetent cells in the endometrium of fetuses and children. *Hum Reprod*. 2003;18(5):969–75.
43. Arruvito L, Giulianelli S, Flores AC, et al. NK cells expressing a progesterone receptor are susceptible to progesterone-induced apoptosis. *J Immunol*. 2008;180(8):5746–53.
44. Okada H, Nakajima T, Sanezumi M, et al. Progesterone enhances interleukin-15 production in human endometrial stromal cells in vitro. *J Clin Endocrinol Metab*. 2000;85(12):4765–70.
45. Wilkens J, Male V, Ghazal P, et al. Uterine NK cells regulate endometrial bleeding in women and are suppressed by the progesterone receptor modulator asoprisnil. *J Immunol*. 2013;191(5):2226–35.
46. Keskin DB, Allan DS, Rybalov B, et al. TGF β promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. *Proc Natl Acad Sci U S A*. 2007;104(9):3378–83.
47. Hanna J, Wald O, Goldman-Wohl D, et al. CXCL12 expression by invasive trophoblasts induces the specific migration of CD16- human natural killer cells. *Blood*. 2003;102(5):1569–77.
48. Quenby S, Bates M, Doig T, et al. Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Hum Reprod*. 1999;14(9):2386–91.
49. Sacks G, Yang Y, Gowen E, et al. Detailed analysis of peripheral blood natural killer cells in women with repeated IVF failure. *Am J Reprod Immunol*. 2012;67(5):434–42.
50. Seshadri S, Sunkara SK. Natural killer cells in female infertility and recurrent miscarriage: A systematic review and meta-analysis. *Hum Reprod Update*. 2014;20(3):429–38.
51. Tang AW, Alfirevic Z, Quenby S. Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: A systematic review. *Hum Reprod*. 2011;26(8):1971–80.
52. Colucci F. The role of KIR and HLA interactions in pregnancy complications. *Immunogenetics*. 2017;69(8–9):557–65.
53. Yang X, Yang Y, Yuan Y, et al. The roles of uterine natural killer (NK) cells and KIR/HLA-c combination in the development of preeclampsia: A systematic review. *Biomed Res Int*. 2020;2020:4808072.
54. Hiby SE, Walker JJ, O'Shaughnessy KM, et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med*. 2004;200(8):957–65.
55. Lash GE, Robson SC, Bulmer JN. Review: Functional role of uterine natural killer (uNK) cells in human early pregnancy decidua. *Placenta*. 2010;31 Suppl:S87–92.
56. Biswas Shivhare S, Bulmer JN, Innes BA, et al. Menstrual cycle distribution of uterine natural killer cells is altered in heavy menstrual bleeding. *J Reprod Immunol*. 2015;112:88–94.
57. Su MT, Lin SH, Chen YC, et al. Genetic association studies of ACE and PAI-1 genes in women with recurrent pregnancy loss: A systematic review and meta-analysis. *Thromb Haemost*. 2013;109(1):8–15.
58. Otun HA, Lash GE, Innes BA, et al. Effect of tumour necrosis factor-alpha in combination with interferon-gamma on first trimester extravillous trophoblast invasion. *J Reprod Immunol*. 2011;88(1):1–11.
59. Lash GE, Otun HA, Innes BA, et al. Inhibition of trophoblast cell invasion by TGFB1, 2, and 3 is associated with a decrease in active proteases. *Biol Reprod*. 2005;73(2):374–81.
60. Lash GE, Otun HA, Innes BA, et al. Regulation of extravillous trophoblast invasion by uterine natural killer cells is dependent on gestational age. *Hum Reprod*. 2010;25(5):1137–45.
61. Parham P. NK cells and trophoblasts: Partners in pregnancy. *J Exp Med*. 2004;200(8):951–5.
62. Hviid TV. HLA-g genotype is associated with fetoplacental growth. *Hum Immunol*. 2004;65(6):586–93.
63. Shobu T, Sageshima N, Tokui H, et al. The surface expression of HLA-f on decidual trophoblasts increases from mid to term gestation. *J Reprod Immunol*. 2006;72(1–2):18–32.
64. Lepin EJ, Bastin JM, Allan DS, et al. Functional characterization of HLA-f and binding of HLA-f trimers to ILT2 and ILT4 receptors. *Eur J Immunol*. 2000;30(12):3552–61.
65. Colonna M, Samardis J. Cloning of immunoglobulin-superfamily members associated with HLA-c and HLA-b recognition by human natural killer cells. *Science*. 1995;268(5209):405–8.
66. Biassoni R, Cantoni C, Falco M, et al. The human leukocyte antigen (HLA)-c-specific “activatory” or “inhibitory” natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med*. 1996;183(2):645–50.
67. Vilches C, Parham P. KIR: Diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol*. 2002;20:217–51.
68. Hong Y, Wang X, Lu P, et al. Killer immunoglobulin-like receptor repertoire on uterine natural killer cell subsets in women with recurrent spontaneous abortions. *Eur J Obstet Gynecol Reprod Biol*. 2008;140(2):218–23.
69. Moffett A, Chazara O, Colucci F, et al. Variation of maternal KIR and fetal HLA-c genes in reproductive failure: Too early for clinical intervention. *Reprod Biomed Online*. 2016;33(6):763–9.
70. Mandelboim O, Reyburn HT, Vales-Gomez M, et al. Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen c alleles and also occurs with empty major histocompatibility complex molecules. *J Exp Med*. 1996;184(3):913–22.
71. Winter CC, Gumperz JE, Parham P, et al. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-c allotype recognition. *J Immunol*. 1998;161(2):571–7.
72. Alecsandru D, Garrido N, Vicario JL, et al. Maternal KIR haplotype influences live birth rate after double embryo transfer in IVF cycles in patients with recurrent miscarriages and implantation failure. *Hum Reprod*. 2014;29(12):2637–43.
73. Hiby SE, Apps R, Sharkey AM, et al. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Investig*. 2010;120(11):4102–10.
74. Hiby SE, Apps R, Chazara O, et al. Maternal KIR in combination with paternal HLA-C2 regulate human birth weight. *J Immunol*. 2014;192(11):5069–73.
75. Pecks U, Maass N, Neulen J. Oocyte donation. *Dtsch Ärztebl Int*. 2011;108(3):23–31.
76. Masoudian P, Nasr A, de Nanassy J, et al. Oocyte donation pregnancies and the risk of preeclampsia or gestational hypertension: A systematic review and metaanalysis. *Am J Obstet Gynecol*. 2016;214(3):328–39.
77. Morin SJ, Treff NR, Tao X, et al. Combination of uterine natural killer cell immunoglobulin receptor haplotype and trophoblastic HLA-c ligand influences the risk of pregnancy loss: A retrospective cohort analysis of direct embryo genotyping data from euploid transfers. *Fertil Steril*. 2017;107(3):677–83.e2.
78. Saito S, Takeda Y, Sakai M, et al. The incidence of pre-eclampsia among couples consisting of Japanese women and Caucasian men. *J Reprod Immunol*. 2006;70(1–2):93–8.
79. Quenby S, Nik H, Innes B, et al. Uterine natural killer cells and angiogenesis in recurrent reproductive failure. *Hum Reprod*. 2008;24(1):45–54.

80. Chen X, Liu Y, Cheung WC, et al. Increased expression of angiogenic cytokines in CD56+ uterine natural killer cells from women with recurrent miscarriage. *Cytokine*. 2018;110:272–6.
81. Chen X, Man GCW, Liu Y, et al. Physiological and pathological angiogenesis in endometrium at the time of embryo implantation. *Am J Reprod Immunol*. 2017;78(2).
82. Liu L, Huang J, Li TC, et al. The effect of elevated progesterone levels before oocyte retrieval in women undergoing ovarian stimulation for IVF treatment on the genomic profile of peri-implantation endometrium. *J Reprod Immunol*. 2017;121:17–25.
83. Mowbray JF, Liddell H, Underwood J, et al. Controlled trial of treatment of recurrent spontaneous abortion by immunisation with paternal cells. *Lancet*. 1985;325(8435):941–3.
84. Wong LF, Porter TF, Scott JR. Immunotherapy for recurrent miscarriage. *Cochrane Database Syst Rev*. 2014;2014(10):CD000012.
85. Dan S, Wei W, Yichao S, et al. Effect of prednisolone administration on patients with unexplained recurrent miscarriage and in routine intracytoplasmic sperm injection: A meta-analysis. *Am J Reprod Immunol*. 2015;74(1):89–97.
86. Cooper S, Laird SM, Mariee N, et al. The effect of prednisolone on endometrial uterine NK cell concentrations and pregnancy outcome in women with reproductive failure. A retrospective cohort study. *J Reprod Immunol*. 2019;131:1–6.
87. Motteram C, Vollenhoven B, Hope N, et al. Live birth rates after combined adjuvant therapy in IVF-ICSI cycles: A matched case-control study. *Reprod Biomed Online*. 2015;30(4):340–8.
88. Woon EV, Day A, Bracewell-Milnes T, et al. Immunotherapy to improve pregnancy outcome in women with abnormal natural killer cell levels/activity and recurrent miscarriage or implantation failure: A systematic review and meta-analysis. *J Reprod Immunol*. 2020;142:103189.
89. Achilli C, Duran-Retamal M, Saab W, et al. The role of immunotherapy in in vitro fertilization and recurrent pregnancy loss: A systematic review and meta-analysis. *Fertil Steril*. 2018;110(6):1089–100.
90. Pignatelli P, Ettorre E, Menichelli D, et al. Seronegative antiphospholipid syndrome: Refining the value of “non-criteria” antibodies for diagnosis and clinical management. *Haematologica*. 2020;105(3):562–72.
91. Petri M. Antiphospholipid syndrome. *Transl Res*. 2020;225:70–81.
92. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost*. 2006;4(2):295–306.
93. Rodrigues VO, Soligo A, Pannain GD. Antiphospholipid antibody syndrome and infertility. *Rev Bras Ginecol Obstet*. 2019;41(10):621–7.
94. Deroux A, Dumestre-Perard C, Dunand-Faure C, et al. Female infertility and serum auto-antibodies: A systematic review. *Clin Rev Allergy Immunol*. 2016;53(1):78–86.
95. D’Ippolito S, Ticconi C, Tersigni C, et al. The pathogenic role of autoantibodies in recurrent pregnancy loss. *Am J Reprod Immunol*. 2020;83(1):e13200.
96. Reimand K, Talja I, Metsküla K, et al. Autoantibody studies of female patients with reproductive failure. *J Reprod Immunol*. 2001;51(2):167–76.
97. Szeliga A, Calik-Ksepka A, Maciejewska-Jeske M, et al. Autoimmune diseases in patients with premature ovarian insufficiency-our current state of knowledge. *Int J Mol Sci*. 2021;22(5).
98. Dhillon-Smith RK, Coomarasamy A. TPO antibody positivity and adverse pregnancy outcomes. *Best Pract Res Clin Endocrinol Metab*. 2020;34(4):101433.
99. Zhong Y-p, Ying Y, Wu H-t, et al. Relationship between antithyroid antibody and pregnancy outcome following in vitro fertilization and embryo transfer. *Int J Med Sci*. 2012;9(2):121–5.
100. He H, Jing S, Gong F, et al. Effect of thyroid autoimmunity per se on assisted reproduction treatment outcomes: A meta-analysis. *Taiwan J Obstet Gynecol*. 2016;55(2):159–65.
101. Dong AC, Morgan J, Kane M, et al. Subclinical hypothyroidism and thyroid autoimmunity in recurrent pregnancy loss: A systematic review and meta-analysis. *Fertil Steril*. 2020;113(3):587–600.e1.
102. Kim NY, Cho HJ, Kim HY, et al. Thyroid autoimmunity and its association with cellular and humoral immunity in women with reproductive failures. *Am J Reprod Immunol*. 2011;65(1):78–87.
103. Marder W, Littlejohn EA, Somers EC. Pregnancy and autoimmune connective tissue diseases. *Best Pract Res Clin Rheumatol*. 2016;30(1):63–80.
104. Alecsandru D, Barrio A, Andia V, et al. Pancreatic autoimmunity: An unknown etiology on patients with assisted reproductive techniques (ART)-recurrent reproductive failure. *PLoS One*. 2018;13(10):e0203446.
105. Alecsandru D, Lopez-Palacios N, Castano M, et al. Exploring undiagnosed celiac disease in women with recurrent reproductive failure: The gluten-free diet could improve reproductive outcomes. *Am J Reprod Immunol*. 2020;83(2):e13209.
106. Andreoli L, Fredi M, Nalli C, et al. Pregnancy implications for systemic lupus erythematosus and the antiphospholipid syndrome. *J Autoimmun*. 2012;38(2-3):J197–J208.
107. Erlebacher A. Immunology of the maternal-fetal interface. *Annu Rev Immunol*. 2013;31(1):387–411.
108. Alecsandru D, Garcia Velasco JA. The excessive presence (percentage or number) of endometrial immune cells in patients with chronic endometritis cannot be associated with reduced endometrial receptivity or recurrent pregnancy failure. *Fertil Steril*. 2020;113(1):85–6.
109. Navti OB, Al-Belushi M, Konje JC. Cytomegalovirus infection in pregnancy – An update. *Eur J Obstet Gynecol*. 2021;258:216–22.
110. Lin X, Chen Y, Fang Z, et al. Effects of cytomegalovirus infection on extravillous trophoblast cells invasion and immune function of NK cells at the maternal-fetal interface. *J Cell Mol Med*. 2020;24(19):11170–6.
111. Stagno S, Pass RF, Cloud G, et al. Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. *JAMA*. 1986;256(14):1904–8.
112. Raschetti R, Vivanti AJ, Vauloup-Fellous C, et al. Synthesis and systematic review of reported neonatal SARS-CoV-2 infections. *Nat Commun*. 2020;11(1):5164.
113. Schwartz DA, Baldewijns M, Benachi A, et al. Chronic histiocytic intervillitis with trophoblast necrosis is a risk factor associated with placental infection from coronavirus disease 2019 (COVID-19) and intrauterine maternal-fetal severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) transmission in live-born and stillborn infants. *Arch Pathol Lab Med*. 2021;145(5):517–28.
114. Wastnedge EAN, Reynolds RM, van Boeckel SR, et al. Pregnancy and COVID-19. *Physiol Rev*. 2021;101(1):303–18.
115. Horton J, Sterrenburg M, Lane S, et al. Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: A systematic review and meta-analysis. *Hum Reprod Update*. 2019;25(5):593–633.
116. Vallvé-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. *Hum Reprod Update*. 2019;25(5):565–92.
117. Kitawaki J, Xu B, Ishihara H, et al. Association of killer cell immunoglobulin-like receptor genotypes with susceptibility to endometriosis. *Am J Reprod Immunol*. 2007;58(6):481–6.
118. Franaszak JM, Alecsandru D, Forman EJ, et al. A review of the pathophysiology of recurrent implantation failure. *Fertil Steril*. 2021;116(6):1436–48.
119. Palomba S, Piltonen TT, Giudice LC. Endometrial function in women with polycystic ovary syndrome: A comprehensive review. *Hum Reprod Update*. 2021;27(3):584–618.

MONITORING OF STIMULATED CYCLES IN ASSISTED REPRODUCTION

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Introduction

Ovarian stimulation is one of the milestones of assisted reproductive techniques (ART). It is defined as a pharmacological treatment aiming at inducing the development of multiple ovarian follicles. It is commonly used for two purposes:

1. For timed intercourse or insemination (IUI), to guide the growth of one or two follicles;
2. In *in vitro* fertilization cycles (IVF), to obtain multiple oocytes at follicular aspiration [1]. Since the birth of IVF in 1978, ART has evolved and the use of natural cycles has very soon been replaced by ovarian stimulation protocols to optimize the results of the technique. The main goal of controlled ovarian stimulation (COS) is to obtain a large number of mature oocytes that can be fertilized resulting in a cohort of embryos. This strategy has substantially increased pregnancy rates in patients because more oocytes are obtained per cycle and, presumably, a greater embryo selection for transfer becomes possible [2]. Ovarian stimulation was therefore aimed at solving two problems: one was the elimination of the risk of having no oocyte at all. The other was the urge to improve efficiency by obtaining several embryos and replacing the best quality embryo to improve the probability of pregnancy.

According to the last NICE guidelines on assessment and treatment of fertility problems, every woman undergoing ovarian stimulation with gonadotropins or clomiphene citrate should be offered ultrasound monitoring [3]. The objectives of monitoring ovarian stimulation cycles are as follows:

1. In timed intercourse or intrauterine insemination, ovarian stimulation cycles are monitored to properly evaluate the timing of the procedure, and to minimize the risk of multiple pregnancy. The simultaneous development of multiple follicles should be communicated to the patient, and the implications of a multiple pregnancy carefully discussed with the couple. Intercourse or insemination should be discouraged if a number of follicles equal to three or more have been recruited [4].
2. In IVF cycles, ovarian stimulation cycles are monitored to estimate the risk of ovarian hyperstimulation syndrome (OHSS) whilst achieving the optimal ovarian response needed for assisted reproduction treatment. For clinicians, the objective of monitoring ovarian stimulation is also to observe which patients have an inadequate response and who is at risk of cycle cancellation [5].

Pre-stimulation assessment

Patients requiring ART treatment are heterogeneous; therefore, it is important to make a good pre-treatment assessment.

Ultrasound

A “baseline” ultrasound should be performed before the start of ovarian stimulation. Its purpose is to evaluate the uterus, the ovaries, and the other pelvic organs before the start of the treatment. This baseline evaluation will then allow the physician to compare ultrasounds that are performed throughout the stimulation process to the initial exam.

The principle criteria to look at during the baseline ultrasound are:

- The orientation of the uterus
- The thickness of the uterus lining
- The measurement of any follicles
- The presence of uterine fibroids, polyps, or ovarian cysts

Although the exact day that a baseline ultrasound is performed may vary slightly for each patient, it is generally scheduled around the time that the patient's period is expected, or around day one or two of the menstrual cycle. If the ultrasound is regular, COS can be started right after. On the contrary, if there are ovarian cysts present, the treatment is commonly postponed until they are reabsorbed.

Another important evaluation which can be performed during the baseline ultrasound is the antral follicle count (AFC). The AFC is the best pre-treatment predictor of follicular response to gonadotropin stimulation during IVF cycles. Depending on the expected ovarian response to stimulation, the objectives of the treatment can be defined in advance, providing more reliable information to the patient about the prognosis and facilitating counselling about the process. On the other hand, clinicians can adopt therapeutic strategies specific to each patient, selecting a personalized protocol. Its measurement could in fact help to select the most appropriate starting gonadotropin dose during IVF treatment [6]. A high number of studies have investigated the role of AFC in the prediction of ovarian response to ovarian stimulation. A high predictive power of AFC in predicting both a poor response and a high response was shown; furthermore, it has been demonstrated that AFC has an added value to female age alone in the prediction of ovarian response [5].

Hormonal assessment

Oestradiol

Assessment of oestradiol at initiation of stimulation is frequently performed in IVF/ICSI and an elevated level usually signifies the presence of a simple follicular cyst, which is then confirmed at ultrasound. Serum oestradiol level below 50 pg/mL confirms the absence of ovarian cysts. Basal oestradiol has also been studied as a predictor of ovarian response to ovarian stimulation. A systematic review over 3911 patients has shown its low accuracy in the prediction of a poor response [7]. Further studies conducted over the same topic [8–12] have confirmed the low accuracy of basal oestradiol in predicting ovarian response [5]. No recommendation can therefore be given in view of the total lack of evidence on

the prognostic role of baseline oestradiol in women undergoing ovarian stimulation for IVF/ICSI.

Progesterone

In a proportion of patients, progesterone remains elevated at menstruation. Elevated progesterone levels at the intended starting date of ovarian stimulation could be associated with reduced pregnancy rates. The proportion of patients with elevated serum progesterone at menstruation varies according to the considered cut-off value. In literature it is reported a prevalence of elevated day 2 serum progesterone of 5% in a cohort study published in 2004 when 1.6 ng/mL was used as the cut-off value [13] and 13.3% when 1.5 ng/mL was taken as the cut-off in a more recent study [14]. A meta-analysis combining three prospective cohort studies on more than 1000 women reported that elevated progesterone level on cycle day 2 prior to initiation of stimulation is associated with a 15% decreased probability of ongoing pregnancy in patients treated by gonadotropins and GnRH antagonist for IVF [14]. Assessment of progesterone prior to initiation of stimulation on cycle day 2 in women undergoing ovarian stimulation with GnRH antagonist and gonadotropins may therefore be beneficial to identify cases with a lower than normal probability of pregnancy. The currently available evidence, however, is not solid, and the clinical value of this test was not assessed.

Assessment during ovarian stimulation

Traditional monitoring of ovarian stimulation during *in vitro* fertilization has included transvaginal ultrasonography (TVUS) plus serum oestradiol levels. However, the effective necessity of the combined approach is controversial and it has been widely debated in the literature. It has been suggested that combined monitoring is time-consuming, expensive, and inconvenient for women, and that simplification of IVF and ICSI therapy by using TVUS only should be considered [5].

Ultrasound

Every woman undergoing ovarian stimulation with gonadotropins or clomiphene citrate should be offered ultrasound monitoring [3]. Follicular growth is monitored by serial ultrasound investigations, which take the name of follicular tracking. The number, volume, and size of each growing follicle should be measured as an integral part of the stimulation treatment. These controls allow the pharmacological dosage to be modulated according to the response obtained. When one or more follicles with a diameter greater than 17–18 mm are displayed, the final maturation of the oocytes contained in the follicles is induced (35–36 hours before the oocyte collection).

Ultrasound techniques can be differentiated by 2- or 3-dimensional techniques, and follicles can be measured both in terms of diameter and volume by both of them. Both 2- and 3-dimensional ultrasound have advantages and limits. Two-dimensional (2D) ultrasound (US) is the most commonly used technique, being available in most centres. However, follicular assessment utilizing 2D US imaging has many challenges, inconsistencies, and irregularity from user to user. The induction of multiple follicular growth during COS, in fact, results in undesirable side effects. Especially when multiple follicles are present, the follicles almost never exhibit a spherical shape, which can result in an overestimation of the mean follicular diameter. The mean diameter estimated by 2D US, in fact, reflects the follicle volume only if the follicles have a round or polygonal shape. In contrast, for elliptical

follicles, the volumes could not be predicted and are commonly over- or underestimated.

Three-dimensional US appears to be superior than 2D US for follicular volume measurement, since a discrepancy of up to 1 mL of the true volume has been shown at oocyte retrieval procedure for the former, and an overestimation of 3.5 mL or underestimation of 2.5 mL of the true volume was observed with the latter. Moreover, significant inter-observer and intra-observer variability contribute to potential discrepancies during follicular assessment using 2D ultrasound [15], which might lead to incorrect timing of human chorionic gonadotropin (hCG) or GnRH agonist administration and consequent worst pregnancy rates. The total number of follicles moreover could not be certainly determined by the classical 2D US. In cases of ovarian hyperstimulation syndrome, it is often difficult to ensure that every follicle is accounted for, and often the qualification regarding the follicle size and quantification is unreliable. Although 3D US systems had been developed and patented by the end of the 1980s, they are not as diffused as 2D systems and there is still a broad debate on the benefits of 3D US in follicle monitoring during COS. A software named SonoAVC has been used to provide automated measurements of follicle size from the stored 3D data sets [16]. SonoAVC helps to identify and measure follicles within a 3D volume. It standardizes the process of follicular assessment and decreases inter-observer and intra-observer variability, while increasing the efficiency of ultrasound follicular monitoring by eliminating the need to measure each individual follicle. Although this technique appears promising and may have implications for the work flow within an IVF centre, timing final follicle maturation and oocyte retrieval on the basis of such automated measures does not appear to improve the clinical outcome of ART.

Endometrial thickness

Human endometrium has a key role in the implantation process, since an adequate endometrial development is required for pregnancy to occur. Thin endometrium on ultrasound during ovarian stimulation has been thought to be associated with poor success rates after IVF, even in the absence of prior intrauterine surgery or infection. The incidence of a thin endometrium (endometrial thickness ≤ 7 mm) is nevertheless low, varying in literature from 2.4% [17] to 11% [18]. There are no studies comparing monitoring endometrial thickness (EMT) compared to no monitoring. However, a large retrospective cohort study (3319 women) reported significant thicker EMT on the hCG day in the clinical pregnancy group compared with the non-pregnant group [19]. In contrast, a large prospective study in 435 women reported no difference in EMT between pregnant and non-pregnant patients [20]. Routine monitoring of EMT during ovarian stimulation is therefore not recommended. A single measurement of the endometrium during ultrasound assessment on the day of triggering or oocyte pick-up could be useful to counsel patients on potential lower chance of pregnancy.

Hormonal assessment

Unlike gonadotropins, steroid hormones are commonly evaluated during COS since they directly reflect the dynamics of follicular growth in the ovaries. Serum oestradiol levels can be useful in evaluating follicular maturity before triggering ovulation. The measurement of progesterone levels before triggering can help in detecting early rise of progesterone levels, which can have a detrimental impact on endometrial receptivity. Steroids are also involved in the implantation process, which is crucial in determining the outcome of ART treatments.

Oestradiol

Serum oestradiol (E2) levels are correlated to the stage of follicular development, since it is produced by the growing follicles when they reach the cut-off of 11 mm in diameter. The amount of oestrogen produced by the dominant follicle increases as it grows, and there is a linear correlation between follicular diameter and E2 levels [21]. The total serum oestradiol at a given moment in the cycle reflects the state of maturity of all follicles present at that time. Therefore, monitoring E2 during ovarian stimulation could be useful to predict the response to COS. The optimum levels of oestradiol, nonetheless, cannot be defined, since they are different from protocol to protocol.

- When a GnRH long agonist protocol is used, downregulation is defined by serum E2 levels below 50 pg/mL. Since the expected increase of serum oestradiol is by 50% per day, an optimal response can be defined as an increase of E2 levels after six days of gonadotropins. On the contrary, low serum E2 values after the first few days of stimulation have been associated with poor outcome and higher cancellation rates. Thus, a better outcome of *in vitro* fertilization may be expected when serum E2 starts early in the cycle and adopts a moderate growth rate [22]. A plateau in plasma E2 for more than three days suggests a poor response.
- When a GnRH-antagonist protocol is used, the addition of the GnRH antagonist to inhibit the LH surge can cause a plateau or a decrease in serum E2 levels. These variations do not compromise the cycle outcome. The E2 value does not help to adjust the dose of gonadotropins after administration of the antagonist. In good outcome cycles, there is a continuous rise in E2 levels until hCG is administered. On the contrary, in cycles which end with no pregnancy, E2 levels show a plateau on the day before hCG administration, which suggests that luteinization or atresia of the more advanced follicles had commenced spontaneously. A value of 100–200 pg/mL per dominant follicle suggests adequate response [23]. A high serum E2 concentration on the day of hCG trigger has been suggested as a predictor of OHSS.

There is a wide variety of reported E2 serum levels in literature above which there is a considerable risk of OHSS. Most of the studies selected an E2 of 3000 pg/mL as a threshold; however, applying this E2 threshold seems to only predict one-third of the total OHSS cases [24, 25]. The number of follicles on the day of hCG administration has been said to be a better predictor of severe OHSS than E2 levels. The predictive value of the threshold of ≥ 13 follicles ≥ 11 mm on the day of hCG has been shown to be statistically significantly superior to the optimal threshold of 2560 ng/L for E2 concentrations in identifying patients at risk for OHSS [25]. Recently, the optimal threshold of 19 follicles ≥ 11 mm on the day of hCG to identify patients at risk of moderate and severe OHSS was found, having a better prognostic value than E2 [26]. Since the etiopathogenesis of OHSS is based more on vascular endothelial growth factor (VEGF) than on E2, the number of follicles is probably a better predictor of OHSS than E2 levels, because OHSS develops due to VEGF production of the follicles rather than their E2 production [25].

There is a wide debate in literature about whether it is fundamental or not to add hormonal assessment to US monitoring in terms of efficacy and safety. A Cochrane meta-analysis on monitoring of ovarian stimulation in IVF/ICSI with ultrasound

alone compared to ultrasound plus serum oestradiol concentration combining six RCTs including 781 women [27] showed that oestradiol measurements plus US did not appear to decrease the probability of OHSS, nor increase the probability of clinical pregnancy or the number of oocytes retrieved compared to US alone.

Serum oestradiol and endometrial receptivity

It is well known that the success of embryonic implantation relies on a perfect dialogue between good quality embryos and a receptive endometrium. During COS there are supra-physiologic levels of steroid hormones produced by the growing ovarian follicles, which induce relevant changes in endometrial receptivity. These changes are detrimental, since uterine receptivity is shown to be deteriorated during COS compared with hormone replacement therapy and natural cycles [28]. E2 concentrations above 3000 pg/mL the day of hCG administration have a deleterious effect on implantation, not only in high-responder patients but also in normal-responder patients [29]. It has been proposed that high E2 levels impair endometrial receptivity instead of oocyte quality because fertilization rate and embryo cleavage (until day 2) in patients with a high response are normal. Indeed, the quality of embryos and the implantation rate in recipients of embryos derived from oocytes of high responders are similar to those in normal responders [30].

Progesterone

Despite an effective suppression of endogenous gonadotropins by GnRH analogues, a small increment in serum progesterone (P) levels has been reported in 5%–30% of COS cycles before hCG administration [31–34]. The origin of this premature elevation of serum P cannot be explained by luteinization of granulosa cells, since endogenous LH levels are low due to suppression by GnRH analogues. Some studies have shown a positive correlation between P levels and some variables in the COS. A positive correlation has been observed with the administered FSH dose [35] and with a longer stimulation period [31]. Moreover, P increase is correlated with a high ovarian response, as it was demonstrated recently that patients with high E2 concentrations and a great number of follicles on the day of hCG have significantly higher P concentrations [36, 37]. It has been widely demonstrated that serum P elevation at ovulation trigger has a negative impact on embryo implantation and therefore on cycle outcome [34, 36, 38]. The cut-off point beyond which P serum value could affect pregnancy implantation is nevertheless controversial. A serum P level ≥ 1.5 ng/mL on the last day of COS has been said to lead to a significant decrease in the ongoing pregnancy rate, irrespective of the GnRH analogue used for pituitary suppression [39]. Nevertheless, it seems that in high responders, the detrimental threshold could be higher [37, 40, 41]. In these patients, the negative impact of premature P elevation has less of an impact on pregnancy rate than in other patients. Probably the negative effect of elevated P is outweighed by other factors with a positive effect in high responders. They may have better and faster developing embryos, which can keep up with endometrial advancement due to premature P elevation [42].

Serum progesterone and endometrial receptivity

Progesterone plays an important role during the luteal phase, particularly in creating decidualization changes needed for implantation and progression of pregnancy. The mechanism underlying the deleterious effect of an elevated P level seems related to the endometrial receptivity rather than oocyte quality [43]. It has

been proposed that in COS cycles, there is an abnormal accelerated endometrial maturation due to the exposure to supra-physiologic concentrations of P in the late follicular phase of IVF cycles [44]. This endometrial advancement anticipates the window of implantation in which the endometrial epithelium acquires a functional ability to support blastocyst adhesion [45, 46]. Women with late follicular phase P levels ≥ 1.5 ng/mL have shown substantially different gene expression profiles than women with normal P levels. It is therefore recommended to monitor P levels, especially during late follicular phase of a COS cycle. It is advisable to vitrify all the embryos for a deferred transfer when P is elevated, because P elevation does not seem to affect frozen-thawed transfer of embryos obtained in the index cycle [40, 47].

Conclusions

Adequate monitoring of COS through US is essential. Steroid measurement could be helpful to control stimulation. As we have described in this chapter, too high E2 and an early P increase have an impact on cycle outcome. On one hand, although the growing follicles can be visualized by ultrasound, E2 production by granulosa cells also reflects the maturation of oocytes. Combined monitoring has been almost universally practiced. Some studies postulated that E2 monitoring is not essential since mature oocyte yield was not improved over monitoring follicle size alone [48]. However, Orvieto [23] suggests that serum E2 level per oocyte is predictive of pregnancy rate per cycle. Moreover, even if combined monitoring with E2 levels does not improve cycle outcome, it would still be valuable until it is proven that OHSS can be avoided without hormonal monitoring [27]. Regarding serum P levels, its measurement helps us to detect an early increase, before triggering, which has a negative impact on endometrial receptivity. If this event occurs, it is recommended to vitrify all the embryos and defer the transfer to a subsequent cycle when endometrial receptivity will not be compromised by elevated P as in the stimulated cycle.

References

- Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, Rienzi L, Sunde A, Schmidt L, Cooke ID, Simpson JL, van der Poel S. The international glossary on infertility and fertility care, 2017. *Fertil Steril*. 2017;108(3):393–406. doi: [10.1016/j.fertnstert.2017.06.005](https://doi.org/10.1016/j.fertnstert.2017.06.005).
- Templeton A, Morris JK. Reducing the risk of multiple births by transfer of two embryos after in vitro fertilization. *N Engl J Med*. 1998 Aug 27;339(9):573–7.
- National Collaborating Centre for Women's and Children's Health (UK). Fertility: Assessment and Treatment for People with Fertility Problems. London (UK): RCOG Press, 2004 Feb.
- van Rumste MM, Custers IM, van der Veen F, van Wely M, Evers JL, Mol BW. The influence of the number of follicles on pregnancy rates in intrauterine insemination with ovarian stimulation: A meta-analysis. *Hum Reprod Update*. 2008;14(6):563–70. doi: [10.1093/humupd/dmn034](https://doi.org/10.1093/humupd/dmn034).
- Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, Kolibianakis E, Kunicki M, Marca L, Lainas A, Clef L, Massin N, Mastenbroek N, Polyzos S, Sunkara N, Timeva SK, Töyli T, Urbancsek M, Vermeulen J, Broekmans N. ESHRE guideline: Ovarian stimulation for IVF/ICSI[®]. *Hum Reprod Open*. 2020;2020(2):hoaa009. doi: [10.1093/hropen/hoaa009](https://doi.org/10.1093/hropen/hoaa009). Erratum in: *Hum Reprod Open*. 2020 Dec 29;2020(4):hoaa067.
- Sylvestre CV, Child T, Pirwany I, Tan SL. Baseline ultrasound and serum hormonal prediction of follicular response to gonadotropin stimulation during IVF. *Fertil Steril*. 2002 Sep 01;78:S6–S7.
- Broekmans FJ, Kwee J, Hendriks DJ, Mol BW, Lambalk CB. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update*. 2006;12(6):685–718. doi: [10.1093/humupd/dml034](https://doi.org/10.1093/humupd/dml034).
- Hendriks DJ, Broekmans FJ, Bancsi LF, de Jong FH, Loosman CW, Te Velde ER. Repeated clomiphene citrate challenge testing in the prediction of outcome in IVF: A comparison with basal markers for ovarian reserve. *Hum Reprod (Oxford, England)*. 2005;20: 163–9.
- Khairy M, Clough A, El-Toukhy T, Coomarasamy A, Khalaf Y. Antral follicle count at down-regulation and prediction of poor ovarian response. *Reprod Biomed Online*. 2008;17:508–14.
- Kwee J, Elting ME, Schats R, McDonnell J, Lambalk CB. Ovarian volume and antral follicle count for the prediction of low and hyper responders with in vitro fertilization. *Reprod Biol Endocrinol*. 2007;5:9.
- Penarrubia J, Peralta S, Fabregues F, Carmona F, Casamitjana R, Balasch J. Day-5 inhibin B serum concentrations and antral follicle count as predictors of ovarian response and live birth in assisted reproduction cycles stimulated with gonadotropin after pituitary suppression. *Fertil Steril*. 2010;94:2590–95.
- van Rooij IA, Broekmans FJ, te Velde ER, Fauser BC, Bancsi LF, de Jong FH, Themmen AP. Serum anti mullerian hormone levels: A novel measure of ovarian reserve. *Hum Reprod (Oxford, England)*. 2002;17:3065–71.
- Kolibianakis EM, Zikopoulos K, Smitz J, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Elevated progesterone at initiation of stimulation is associated with a lower ongoing pregnancy rate after IVF using GnRH antagonists. *Hum Reprod (Oxford, England)*. 2004;19:1525–9.
- Hamidine O, Macklon NS, Eijkemans MJ, Laven JS, Cohlen BJ, Verhoeff A, van Dop PA, Bernardus RE, Lambalk CB, Oosterhuis GJ, et al. Elevated early follicular progesterone levels and in vitro fertilization outcomes: A prospective intervention study and meta-analysis. *Fertil Steril*. 2014;102:448–54.e441.
- Forman RG, Robinson J, Yudkin P, Egan D, Reynolds K, Barlow DH. What is the true follicular diameter: An assessment of the reproducibility of transvaginal ultrasound monitoring in stimulated cycles. *Fertil Steril*. 1991;56:989–92.
- Raine-Fenning N, Jayaprakasan K, Clewes J, Joergner I, Bonaki SD, Chamberlain S, Devlin L, Priddle H, Johnson I. SonoAVC: A novel method of automatic volume calculation. *Ultrasound Obstet Gynecol*. 2008;31(6):691–6. doi: [10.1002/uog.5359](https://doi.org/10.1002/uog.5359).
- Kasius A, Smit JG, Torrance HL, Eijkemans MJ, Mol BW, Opmeer BC, Broekmans FJ. Endometrial thickness and pregnancy rates after IVF: A systematic review and meta-analysis. *Hum Reprod Update*. 2014;20:530–41.
- Coelho Neto MA, Martins WP, Lima ML, Barbosa MA, Nastri CO, Ferriani RA, Navarro PA. Ovarian response is a better predictor of clinical pregnancy rate following embryo transfer than is thin endometrium or presence of an endometrioma. *Ultrasound Obstet Gynecol*. 2015;46:501–5.
- Zhao J, Zhang Q, Wang Y, Li Y. Endometrial pattern, thickness and growth in predicting pregnancy outcome following 3319 IVF cycle. *Reprod Biomed Online*. 2014;29:291–8.
- Zhang T, He Y, Wang Y, Zhu Q, Yang J, Zhao X, Sun Y. The role of three-dimensional power Doppler ultrasound parameters measured on hCG day in the prediction of pregnancy during in vitro fertilization treatment. *Eur J Obstetr Gynecol Reprod Biol*. 2016;203:66–71.
- Loumaye E, Engrand P, Howles CM, O'Dea L. Assessment of the role of serum luteinizing hormone and estradiol response to follicle-stimulating hormone on in vitro fertilization treatment outcome. *Fertil Steril*. 1997;67(5):889–99.
- Dirnfeld M, Lejeune B, Camus M, Vekemans M, Leroy F. Growth rate of follicular estrogen secretion in relation to the outcome of in vitro fertilization and embryo replacement. *Fertil Steril*. 1985;43(3):379–84.

23. Orvieto R, Zohav E, Scharf S, Rabinson J, Meltcer S, Anteby EY, et al. The influence of estradiol/follicle and estradiol/oocyte ratios on the outcome of controlled ovarian stimulation for in vitro fertilization. *Gynecol Endocrinol.* 2007 Feb;23(2):72–5.
24. Aboulghar M. Prediction of ovarian hyperstimulation syndrome (OHSS). Estradiol level has an important role in the prediction of OHSS. *Hum Reprod.* 2003;18(6):1140–1.
25. Papanikolaou EG, Pozzobon C, Kolibianakis EM, Camus M, Tournaye H, Fatemi HM, et al. Incidence and prediction of ovarian hyperstimulation syndrome in women undergoing gonadotropin releasing hormone antagonist in vitro fertilization cycles. *Fertil Steril.* 2006;85:112–20.
26. Griesinger G, Verweij PJ, Gates D, Devroey P, Gordon K, Stegmann BJ, et al. Prediction of ovarian hyperstimulation syndrome in patients treated with corifollitropin alfa or rFSH in a GnRH antagonist protocol. *PLoS One.* 2016 Mar 7;11(3):e0149615.
27. Kwan I, Bhattacharya S, Kang A, Woolner A. Monitoring of stimulated cycles in assisted reproduction (IVF and ICSI). *Cochrane Data Syst Rev.* 2014;2014(8):Cd005289.
28. Paulson RJ, Sauer MV, Lobo RA. Embryo implantation after human in vitro fertilization: Importance of endometrial receptivity. *Fertil Steril.* 1990;53(5):870–4.
29. Simón C, Cano F, Valbuena D, Remohí J, Pellicer A. Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. *Hum Reprod.* 1995;10(9):2432–7.
30. Braga DPAF, Setti AS, Iaconelli A Jr, Borges E Jr. Predictive factors for successful pregnancy in an egg-sharing donation program. *JBRA Assist Reprod.* 2020;24(2):163–9. doi:[10.5935/1518-0557.20190087](https://doi.org/10.5935/1518-0557.20190087).
31. Bosch E, Valencia I, Escudero E, Crespo J, Simón C, Remohí J, et al. Premature luteinization during gonadotropin-releasing hormone antagonist cycles and its relationship with in vitro fertilization outcome. *Fertil Steril.* 2003 Dec;80(6):1444–9.
32. Silverberg KM, Burns WN, Olive DL, Riehl RM, Schenken RS. Serum progesterone levels predict success of in vitro fertilization/embryo transfer in patients stimulated with leuprolide acetate and human menopausal gonadotropins. *J Clin Endocrinol Metab.* 1991;73:797–803.
33. Edelstein MC, Seltman HJ, Cox BJ, Robinson SM, Shaw RA, Muasher SJ. Progesterone levels on the day of human chorionic gonadotropin administration in cycles with gonadotropin-releasing hormone agonist suppression are not predictive of pregnancy outcome. *Fertil Steril.* 1990;54:853–7.
34. Ubaldi F, Albano C, Peukert M, Riethmuller-Winzen H, Camus M, Smitz J, et al. Subtle progesterone rise after the administration of the gonadotrophin-releasing hormone antagonist cetrorelix in intracytoplasmic sperm injection cycles. *Hum Reprod.* 1996;11:1405–7.
35. Filicori M, Cognigni GE, Pocognoli P, Tabarelli C, Spettoli D, Taraborrelli S, et al. Modulation of folliculogenesis and steroidogenesis in women by graded menotropin administration. *Hum Reprod.* 2002;17:2009–15.
36. Kyrou D, Al-Azemi M, Papanikolaou EG, Donoso P, Tziomalos K, Devroey P, et al. The relationship of premature progesterone rise with serum estradiol levels and number of follicles in GnRH antagonist/recombinant FSH-stimulated cycles. *Eur J Obstet Gynecol Reprod Biol.* 2012;162:165–8.
37. Griesinger G, Mannaerts B, Andersen CY, Witjes H, Kolibianakis EM, Gordon K. Progesterone elevation does not compromise pregnancy rates in high responders: A pooled analysis of in vitro fertilization patients treated with recombinant follicle-stimulating hormone/gonadotropin-releasing hormone antagonist in six trials. *Fertil Steril.* 2013;100:1622.
38. Kosmas IP, Kolibianakis EM, Devroey P. Association of estradiol levels on the day of hCG administration and pregnancy achievement in IVF: A systematic review. *Hum Reprod.* 2004;19:2446–53.
39. Bosch E, Labarta E, Crespo J, Simón C, Remohí J, Jenkins J, et al. Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for in vitro fertilization analysis of over 4000 cycles. *Hum Reprod.* 2010;25:2092–100.
40. Xu B, Li Z, Zhang H, Jin L, Li Y, Ai J, et al. Serum progesterone level effects on the outcome of in vitro fertilization in patients with different ovarian response: An Analysis of more than 10,000 cycles. *Fertil Steril.* 2012;97:1321.
41. Requena A, Cruz M, Bosch E, Meseguer M, García-Velasco JA. High progesterone levels in women with high ovarian response do not affect clinical outcomes: A retrospective cohort study. *Reprod Biol Endocrinol.* 2014;12:69.
42. Fatemi HM, Doody K, Griesinger G, Witjes H, Mannaerts B. High ovarian response does not jeopardize ongoing pregnancy rates and increases cumulative pregnancy rates in a GnRH-antagonist protocol. *Hum Reprod.* 2013;28(2):442–52.
43. Melo M, Meseguer M, Garrido N, Bosch E, Pellicer A, Remohí J. The significance of premature luteinization in an oocyte-donation programme. *Hum Reprod.* 2006;21(6):1503–7.
44. Papanikolaou EG, Bourgain C, Kolibianakis E, Tournaye H, Devroey P. Steroid receptor expression in late follicular phase endometrium in GnRH antagonist IVF cycles is already altered, indicating initiation of early luteal phase transformation in the absence of secretory changes. *Hum Reprod.* 2005;20:1541–7.
45. Horcajadas JA, Sharkey AM, Catalano RD, Sherwin JRA, Domínguez F, Burgos LA, et al. Effect of an intrauterine device on the gene expression profile of the endometrium. *J Clin Endocrinol Metab.* 2006;91:3199–207.
46. Bourgain C, Devroey P. The endometrium in stimulated cycles for IVF. *Hum Reprod Update.* 2003;9:515–22.
47. Venetis CA, Kolibianakis EM, Bosdou JK, Tarlatzis BC. Progesterone elevation and probability of pregnancy after IVF: A systematic review and meta-analysis of over 60 000 cycles. *Hum Reprod Update.* 2013;19(5):433–57.
48. Vandekerckhove F, Gerris J, Vansteelandt S, De Sutter P. Adding serum estradiol measurements to ultrasound monitoring does not change the yield of mature oocytes in IVF/ICSI. *Gynecol Endocrinol.* 2014;30(9):649–52.

HOME MONITORING OF ART CYCLES

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Introduction

Ovarian cycle monitoring has been the mainstay of ovarian stimulation for assisted reproductive technology (ART) for nearly 60 years, providing valuable information on ovarian response, on the individualization of treatment plans (e.g. choice of stimulation protocol and need for dose adjustments), and on the optimal timing of ovulation triggering in order to ensure retrieval of the optimal number of oocytes while reducing the risk of ovarian hyperstimulation syndrome (OHSS) [1, 2].

Ovarian cycles can be monitored by transvaginal ultrasound and serum hormone testing. Using ultrasound, a physician can monitor the size and number of the developing follicles, and serum hormone testing can provide a hormone profile at the different stages of ovarian stimulation.

The need for frequent monitoring during ART cycles remains a logistical challenge for both patients and clinic staff [3–5]. Repeat clinic visits are time-consuming and disturb the daily lives of patients. A survey across four European countries showed that 21%–36% of patients reported difficulty with fitting fertility treatments into their lives, and the need for repeated appointments and time off work was a barrier to treatment access [6]. Frequent monitoring is also a resource- and time-intensive use of laboratory and clinic staff [7]. Furthermore, in order to reduce the risk of in-person transmission during the Covid-19 pandemic, fertility clinics started to minimize in-person visits where possible [8].

Clinics have trialled the combination of remote home monitoring and tele-counselling, including self-operated ultrasounds [9–11] and hormone tests on saliva [5], aiming to limit the challenges of frequent monitoring and to improve patient experience. Notwithstanding the potential applicability of remote home monitoring, the concept requires validation in clinical practice.

In this chapter, we reflect on traditional clinic-based monitoring during controlled ovarian stimulation (COS) for ART and on the value of the potential digital health approach combining remote self-operated ultrasound and remote urinary hormone testing with tele-counselling.

Traditional clinic-based ovarian cycle monitoring

Individualizing COS for ART treatment is key to reducing the risk of OHSS in hyper responders [12] and for avoiding cycle cancellations due to inadequate ovarian response in poor responders [1, 13–19]. These individualized treatment decisions may focus on the following: (i) the protocol (e.g. gonadotropin-releasing hormone [GnRH] agonist or antagonist), (ii) the gonadotropin (type, starting dose, and dose adaptation during ovarian stimulation), (iii) the trigger for final oocyte maturation (type and timing), and (iv) luteal phase support (type and duration) [1, 16–18].

At the start of COS for ART, professional guidelines recommend taking account of the characteristics of an individual patient and her hormonal profile, but they differ with regard to the specific recommendations. The guidelines of the European Society of Human Reproduction and Embryology (ESHRE) recommend patient characteristics, such as age and antral follicle count (AFC), and serum markers, including anti-Müllerian hormone (AMH) and follicle-stimulating hormone (FSH), as predictors of ovarian response to guide COS for ART [20]. The American Society for Reproductive Medicine (ASRM) also recommends measurement of serum E2 in combination with basal serum FSH as predictors of ovarian reserve [15, 19]. This is not surprising since basal levels of serum oestradiol (E2) >60–80 pg/mL are known to suppress FSH.

With regard to monitoring during the course of COS for ART, the professional guidelines differ in their recommendations and are not completely in line with the worldwide common practice of combining ultrasound with serum hormonal assays [5, 21–24]. This practice was confirmed by a recent (September to October 2021) open-access cross-sectional survey with 25 multiple-choice questions carried out by IVF worldwide [25]. No fewer than 528 fertility specialists from eight countries filled out the questionnaire. The majority of responders (87.9%) were clinicians with more than 15 years of experience in reproductive medicine, and almost half of them (46.2%) performed more than 500 ART cycles annually. The respondents (98.9%) shared that they predominantly relied on ultrasound to monitor ovarian stimulation during ART cycles, although hormone monitoring was accepted and performed by the vast majority of respondents (79.5%). In particular, both ultrasound monitoring and reproductive hormone monitoring were used by a high proportion of respondents to support decision-making to proceed/not to proceed with a freeze-all cycle for prevention of OHSS (89.2% and 69.6%, respectively), to adjust the gonadotropin dose (81.2% and 61.7%, respectively), and for timing oocyte ovulation triggering (97.5% and 45%, respectively) [25]. Overall, the survey data confirm that ultrasound monitoring supported by reproductive hormone monitoring is common practice during COS for ART today (Figure 49.1). This practice is also in line, overall, with existing international professional guidelines from ESHRE, ASRM, and the World Health Organization (WHO) [20, 26, 27]. The ESHRE 2020 guidelines state that the addition of basal serum E2, progesterone (P4), and luteinizing hormone (LH) monitoring to conventional ultrasound assessments during COS for ART is “probably not recommended” [20]. This was based on a 2014 Cochrane meta-analysis of six randomized controlled trials that showed that adding serum E2 monitoring to ultrasound had no benefit in terms of improving pregnancy rates, number of oocytes retrieved, or detection of OHSS [7]. It should, however, be noted that the majority of patients (>70%) in the Cochrane analysis underwent a GnRH-agonist protocol, so these guidelines are probably mainly applicable to GnRH-agonist protocol cycles [7]; whereas, today, the GnRH-antagonist protocol is recommended over the GnRH protocols, given the comparable

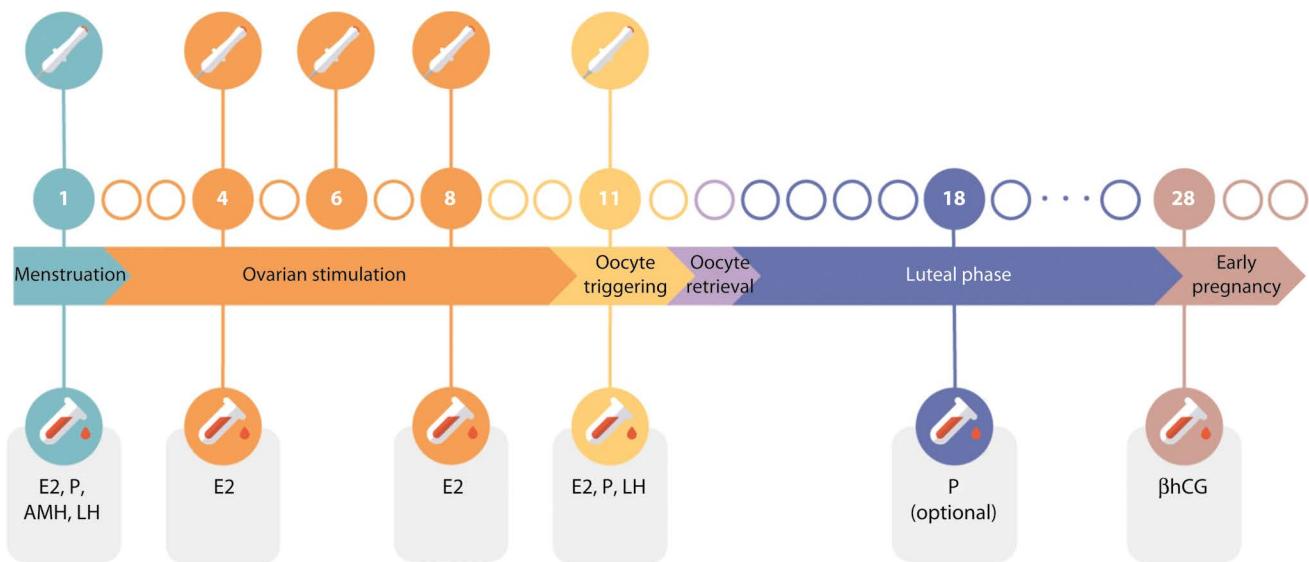


FIGURE 49.1 Typical schedule for the frequency of transvaginal ultrasound (top panel) and serum hormonal monitoring (bottom panel) during a cycle of COS for ART. The numbers in the circles represent the days in a typical stimulation cycle corresponding to the respective phases (only days on which monitoring is typically performed are labelled). Abbreviations: AMH, anti-Müllerian hormone; β hCG, β human chorionic gonadotropin; E2, oestradiol; LH, luteinizing hormone; P, progesterone.

efficacy and higher safety in the *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment [20]. More importantly, the ESHRE guidelines cite the quality of evidence supporting their conclusions as “low,” meaning that shared decision-making is recommended to make appropriate treatment choices for different patients [28]. In contrast, the ASRM 2016 guideline on the prevention and treatment of moderate and severe OHSS considered the evidence for the utility of serum E2 level monitoring as fair [27]. This was based on elevated serum E2 concentrations being associated with an increased risk of OHSS, predicted by an E2 cut-off value of 3500 pg/mL around the day of triggering during COS. Furthermore, an evidence-based review directed by the WHO guideline development group on the global management of COS recommends monitoring E2 in combination with ultrasound during COS in women at high risk of OHSS [26, 27]. Finally, the ESHRE guidelines did recognize the combination of hormonal monitoring and ultrasound as “good practice” for determining the time of ovulation triggering in COS cycles: “The decision on timing of triggering in relation to follicle size is multi-factorial, taking into account the size of the growing follicle cohort, the hormonal data on the day of pursued trigger, duration of stimulation, patient burden, financial costs, experience of previous cycles and organizational factors for the centre” [20].

Monitoring P4 levels to identify “premature luteinization” during COS for ART is a more controversial subject [29]. Kaponis and colleagues suggested the terminology “follicular-phase progesterone rise (FPPR),” as LH is not always the dependent factor and may in fact occur before the day of human chorionic gonadotropin (hCG) [29, 30]. A 2019 survey found that fertility specialists choose to use frozen-warmed embryo transfer cycles to avoid the potential harmful impact of progesterone elevation [31], which may affect both the quality of oocytes and the endometrium [29, 32]. To the best of our knowledge, there is no national or international guideline on the use of elective frozen-warmed embryo transfer based on serum P4 levels during COS.

Patient perspective on traditional clinic-based ovarian cycle monitoring

Few studies focused on the perspective of patients on traditional clinic-based ovarian cycle monitoring by ultrasound or serum hormonal assays. A survey across four European countries showed that 21%–36% of patients reported difficulty with fitting fertility treatments, requiring repeated clinic visits, into their life [6]. Another survey showed that women who needed fewer clinic visits during treatment reported less interference of treatment with their daily life [33]. The fact that repeated clinic visits for ovarian cycle monitoring are usually planned during office hours and that every clinic visit subjects patients to the discomfort of time spent in waiting rooms does not help [34, 35]. On a more positive note, every clinic visit is an opportunity for supportive interactions between staff and patients [34, 35]. Vaginal ultrasounds were reported to cause discomfort in women undergoing ovarian cycle monitoring for fertility preservation, as this required revealing private body parts to professionals [36]. Women considered blood tests a frustrating obligation of fertility treatment [37], which is in line with women feeling anxious about and reporting pain from the subcutaneous injections required for COS [36, 38]. The frequency of venepuncture has even been reported to influence a woman’s choice of fertility medication [39].

The potential digital health approach combining remote ovarian cycle monitoring with tele-counselling

The digital health approach, combining remote self-operated ultrasound, remote hormone testing, and tele-counselling, could potentially alleviate the aforementioned burdens and anxieties. Such an approach could prevent the inconvenience of frequent clinic visits and waiting during office hours, anxiety about

investigations in which private body parts have to be uncovered to staff, and the anxiety and pain caused by venepunctures. In addition, self-monitoring could make patients feel in control rather than feeling a loss of control during fertility treatments [37, 40]. Tele-counselling associated with remote monitoring should aim to provide patients with the much-needed supportive interactions with staff [34, 35, 37, 41, 42]. Video consultations for a second opinion were recently reported as a positive experience by fertility patients [43]. Further studies are required to gain in-depth insight in the efficiency of and patient experience with the combination of remote cycle monitoring and tele-counselling.

Remote self-operated ultrasound: SOET system

Self-operated endovaginal telemonitoring (SOET) is a reusable portable sonographic device connected with an FDA approved, CE-marked endovaginal probe (Figure 49.2) [10]. It was first introduced in 2009 in order to reduce the number of clinic monitoring visits for women undergoing ART [44, 45]. Prior to first use, patients would receive instruction from a midwife, nurse practitioner, or experienced sonography technician to ensure that they are comfortable with performing sonography themselves. Patients would then perform the sonography at home or anywhere that has access to Internet [45]. Home monitoring with SOET was shown to offer several advantages, including greater flexibility of planning for patients and their partners, less income loss due to attending appointments during office hours, and less environmental impact due to reduced travelling [4]. SOET also allowed partners to play a more active role in the treatment process, potentially making the procedure less stressful [3, 45]. Additionally, with SOET reducing the need for in-person sonographies, access to treatment was increased for those where sonography was not available within



FIGURE 49.2 Self-operated endovaginal telemonitoring (SOET) system. SOET is composed of a portable tablet computer and a connected endovaginal probe. (Courtesy of Jan Gerris.)

reasonable travelling distance [45]. Importantly, the goal was not to replace all clinic-based sonography with SOET. Clinic-based sonography would still be available for complicated COS cycles associated with abnormal follicular growth, when ovarian cysts were present, or for anxious patients; and a backup sonography in the clinic could be scheduled if needed [45]. Small-scale prospective studies of ART treatment with SOET monitoring have found similar clinical outcomes to ART treatment with traditional clinic-based monitoring, with the potential for 90% of patients to avoid clinic visits for sonography [10, 11]. A randomized controlled trial showed that conception and ongoing pregnancy rates resulting from SOET-monitored treatment cycles were similar to those from cycles monitored via traditional methods, but with reduced overall costs for payer, patient, and employer [9]. The use of SOET also had a positive impact on patient-reported outcomes (such as feelings of empowerment, discretion, partner involvement, and reduced stress) compared with traditional monitoring. However, the patient-reported outcomes were not directly collected from patients during the study, but were mostly derived from questionnaires developed by the study authors and completed during post-study interviews, thereby introducing a risk of bias [9].

Remote urinary hormone testing

Historically, hormone levels were measured with urinary hormone assays, of which the advantages and disadvantages were summarized in a recent article by Hart et al. [31]. Urinary hormonal assays, assessing the levels of E2 and P4 and their metabolites, estrone-3-glucuronide (E1-3G) and pregnanediol-3-glucuronide (PdG), provide information on ovarian and ovulatory function [46, 47], on follicular growth (a principal parameter in evidencing a successfully induced cycle), and on the timing of ovulation. Although Rapi et al. showed that correlation between E1-3G and follicular size presented a large individual variability, urinary hormone assays were considered a reliable method for detecting the optimal timing of the day of ovulation triggering with hCG during COS for ART treatment [48].

Clinic-based urinary hormone assays, which require sophisticated sample handling techniques [31], have been replaced by serum hormone assays, which have a well-defined reference range and are more amenable for automation. Taking into account technological progress in urinary hormonal assays, remote urinary hormone assays in combination with ultrasound testing might, however, be of value to professionals as they reduce the need for, and associated costs of, trained personnel taking samples.

Recent developments have enabled home testing of urinary E1-3G and LH by patients via point of care (POC) devices [31, 49]. For example, the experiences of 232 patients randomized between home-based urinary LH testing or clinic-based monitoring to predict ovulation in natural and gonadotropin-stimulated cycles in preparation of frozen-thawed embryo transfers were recently assessed [50]. The results suggested that patient experiences were significantly better with home-based monitoring of LH, but regrettably, the reliability of the patient-reported experience measure was not evaluated [50]. Furthermore, a prospective study in 53 women evaluated the ClearPlan® fertility monitoring system, which simultaneously detects LH and E1-3G in early morning urine to delineate three levels of fertility (low, high, and peak, the latter resulting from the surge in LH), to predict ovulation in natural cycles; and the results were compared with traditional ovarian cycle monitoring with transvaginal ultrasound and serum hormone measurements [51]. In 91%

of the cycles studied, the remote urinary monitoring accurately predicted a two-day window for ovulation [51]. The authors concluded that the ClearPlan® fertility monitoring system, which allows retrospective analysis of data stored for several months, could potentially be used as a diagnostic tool and for monitoring during infertility treatment [51]. Finally, a pilot study assessing the use of multi-level pregnancy tests at home, which can monitor changes in hCG after ART, found a good correlation with serum lab results [52]. The assessment, which used a questionnaire without previously proven reliability, suggested that 73% of women reported being “satisfied” or “very satisfied” with the remote monitoring, and that 97% found it “easy” or “very easy” to use [52].

Remote salivary hormone testing

Another non-invasive option for remote hormone monitoring is the possibility to perform salivary tests. So far, it has been shown that salivary E2 correlates well with serum E2, but also that salivary P4 correlates poorly with serum P4 [5]. Another disadvantage is the need to send the saliva samples to a laboratory for enzyme-linked immunosorbent assay, which adds delay to the time for receiving results [5, 53].

Digital health systems and tele-counselling

Remote monitoring devices that negate the need for in-person on-site appointments have the potential to improve access to services [54, 55]. Remote monitoring devices are often used in combination with tele-counselling via devices such as phones, computers, and mobile applications (Figure 49.3). The internet has become an established source of fertility treatment-related information (and misinformation) [56] and for providing personalized information via, for example, mobile applications that aim to improve patient understanding, patient–staff relationships, and adherence to medical advice. Digital health systems combining remote monitoring with tele-counselling are currently implemented in many clinical specialties.

Digital health systems seem of particular benefit for patients with chronic conditions. For example, studies have shown that telephone-administered assessments are effective for monitoring medication adherence, and wearable sensors may improve the timing of treatment, in patients with multiple sclerosis [57, 58]. Additionally, continuous blood glucose monitoring may influence treatment decisions in patients with diabetes, and remote monitoring has proven reliable for measuring pulmonary function in patients with Duchenne muscular dystrophy [59–61].

Digital health systems with connecting urinary hormone assays have great potential in the field of family planning. Hormone monitoring is also commonly used to assess fetal development during early pregnancy to prevent miscarriage [2, 7, 20, 62, 63]. Currently available diagnostic tools for the identification of corpus luteum deficiency, a possible cause of miscarriage, are however imprecise and unreliable; likely due to rapid fluctuations in serum progesterone levels [64]. Other studies have shown that women affected by habitual miscarriage display a significantly higher ratio of E1-3G:PdG in the luteal phase, suggesting that urinary measurements may be less prone to fluctuation and therefore more reliable than serum measurements [65]. Congruent with these findings, other investigators have concluded that urinary assessments, such as LH, PdG, and E1-3G, may aid in the

diagnosis of corpus luteum deficiency and ensure prompt treatment in the event of abnormality [66, 67].

The use of digital health systems has only recently been investigated in the context of ART treatment [68]. For example, one study showed that implementing an electronic patient portal for ART patients reduced total waiting time, increased the number of patients treated, and did not negatively impact treatment outcomes [69]. Another study showed that video consultations for a second opinion are experienced positively by ART patients [43].

Digital health systems proved especially important for ART during the Covid-19 pandemic, as fertility treatment was suspended for all but urgent cases under advice published by ESHRE and ASRM [70]. A one-month discontinuation of IVF in the United States was estimated to result in 369 fewer women having a live birth, mainly due to the increase in age during the shutdown, making women ineligible for treatment [71, 72]. Other evidence also suggests that the pandemic itself had a severe psychological impact on infertile patients, and was a major source of stress and anxiety [73, 74]. This prompted the WHO and the major societies for reproductive medicine to emphasize the importance of sustaining reproductive care during the pandemic, by defining infertility as both a disease and disability [70, 75, 76]. Quick implementation of tele-counselling during the Covid-19 pandemic allowed continued access to care, whilst limiting risk of exposure via reducing in-person appointments [77]. Additionally, patient attitudes towards tele-counselling have changed due to the Covid-19 pandemic, with the experience making patients more likely to opt for video versus in-person consultations [77].

Conclusions

Clinic-based ultrasound and serum hormonal assays are widely used in clinical practice to monitor ovarian cycles, optimize treatment outcomes, and reduce complications during ART treatment. The digital health approach, combining remote self-operated ultrasound, remote hormone testing, and tele-counselling, has enormous potential to add value for professionals and patients, by improving access to care by decreasing the need for in-person clinic visits that are expensive and that disrupt patients' lives.

From a technological standpoint, SOET seems vital for remote monitoring during ART. Patients can take real-time SOET images at their own convenience, which are then sent securely and directly over the internet to the healthcare provider, who can analyse them immediately or at a later time. After analysis, the healthcare provider will send the patient instructions regarding gonadotropin dose, follow-up sonography, and timing of hCG injection, when applicable [3]. SOET seems best suited to patients who have undergone at least one previous ART cycle and who are not adverse to technology [3]. Remote urine testing is also a very interesting potential option, as it can be done at the patient's convenience, and results can be sent to healthcare providers, stored, and interpreted to allow timely treatment decisions, such as real-time dose adjustments and timing of ovulation triggering (Figure 49.3).

At present, more evidence is needed to evaluate the potential value and impact of this novel digital healthcare approach, combining remote self-operated ultrasound, remote hormone testing, and tele-counselling, on the experience of both patients and healthcare professionals, on the quality of treatment decisions



FIGURE 49.3 A hypothetical home-monitoring set-up, demonstrating the bidirectional flow of information and monitoring via dedicated devices and secure IT infrastructure between the patient and physician. The partial replacement of clinic-based monitoring by remote home-based monitoring with self-operated ultrasound and hormone tests on urine may help alleviate the logistical challenges for both patients and clinic staff due to repeated clinic visits, and ease patient discomfort and disruption to patients' lives.

during COS for ART treatment, and ultimately on the cumulative probability of a successful reproductive outcome per started ART cycle. Although it is tempting to speculate that a remote monitoring/digital approach will reduce patient burden and therefore reduce ART treatment discontinuation, high-quality studies are needed using valid and reliable methods to assess patient-reported outcomes and experience measures (e.g. for quality of life and general well-being). In addition, well-designed prospective studies in selected patient populations are needed to support the hypothesis that remote monitoring methods have comparable medical value and reliability compared with traditional methods. Such evidence then requires subsequent confirmation in well-designed real-world studies.

References

1. Lunenfeld B. Gonadotropin stimulation: Past, present and future. *Reprod Med Biol.* 2012;11:11–25. doi: [10.1007/s12522-011-0097-2](https://doi.org/10.1007/s12522-011-0097-2).
2. Lunenfeld B, Donini P. Le traitement de l'anovulation par les hormones gonadotropes humaines. *Annales d' Endocrinologie.* 1964;156.
3. Gerris J. Telemonitoring in IVF/ICSI. *Curr Opin Obstet Gynecol.* 2017;29:160–67. doi: [10.1097/GCO.0000000000000363](https://doi.org/10.1097/GCO.0000000000000363).
4. Gerris J. ART monitoring: An end to frequent clinic visits and needle sticks? In: *Patient-Centered Assisted Reproduction: How to Integrate Exceptional Care with Cutting-Edge Technology*, Domar AD, Sakkas D and Toth TL (eds.). Cambridge: Cambridge University Press, pp. 39–53, 2020.

5. Sakkas D, Howles CM, Atkinson L, et al. A multi-centre international study of salivary hormone oestradiol and progesterone measurements in ART monitoring. *Reprod Biomed Online.* 2021;42:421–8. doi: [10.1016/j.rbmo.2020.10.012](https://doi.org/10.1016/j.rbmo.2020.10.012).
6. Domar A, Gordon K, Garcia-Velasco J, et al. Understanding the perceptions of and emotional barriers to infertility treatment: A survey in four European countries. *Hum Reprod.* 2012;27:1073–9. doi: [10.1093/humrep/des016](https://doi.org/10.1093/humrep/des016).
7. Kwan I, Bhattacharya S, Kang A, et al. Monitoring of stimulated cycles in assisted reproduction (IVF and ICSI). *Cochrane Data Syst Rev.* 2014;2014: Cd005289. doi: [10.1002/14651858.CD005289.pub3](https://doi.org/10.1002/14651858.CD005289.pub3).
8. Robertson I, Kermack AJ, Cheong Y. The impact of covid-19 on infertility services and future directions. *Reprod Fertil.* 2020;1:C3–C7. doi: [10.1530/raf-20-0017](https://doi.org/10.1530/raf-20-0017).
9. Gerris J, Delvigne A, Dhont N, et al. Self-operated endovaginal tele-monitoring versus traditional monitoring of ovarian stimulation in assisted reproduction: An RCT. *Hum Reprod.* 2014;29:1941–8. doi: [10.1093/humrep/deu168](https://doi.org/10.1093/humrep/deu168).
10. Gerris J, Vandekerckhove F, De Sutter P. Outcome of one hundred consecutive ICSI attempts using patient operated home sonography for monitoring follicular growth. *Fact Views Vis Obstgyn.* 2016;8:141–6.
11. Pereira I, von Horn K, Depenbusch M, et al. Self-operated endovaginal telemonitoring: A prospective, clinical validation study. *Fertil Steril.* 2016;106:306–10.e301. doi: [10.1016/j.fertnstert.2016.03.043](https://doi.org/10.1016/j.fertnstert.2016.03.043).
12. Alviggi C, Humaidan P, Ezcurra D. Hormonal, functional and genetic biomarkers in controlled ovarian stimulation: Tools for matching patients and protocols. *Reprod Biol Endocrinol.* 2012;10:9. doi: [10.1186/1477-7827-10-9](https://doi.org/10.1186/1477-7827-10-9).
13. van Tilborg TC, Torrance HL, Oudshoorn SC, et al. Individualized versus standard FSH dosing in women starting IVF/ICSI: An RCT. Part 1: The predicted poor responder. *Hum Reprod.* 2017;32:2496–505. doi: [10.1093/humrep/dex318](https://doi.org/10.1093/humrep/dex318).
14. La Marca A, Blockeel C, Bosch E, et al. Individualized FSH dosing improves safety and reduces iatrogenic poor response while maintaining live-birth rates. *Hum Reprod.* 2018;33:982–3. doi: [10.1093/humrep/dey061](https://doi.org/10.1093/humrep/dey061).
15. Committee on Gynecologic Practice - American Society for Reproductive Medicine. Infertility workup for the Women's health specialist: ACOG committee opinion, number 781. *Obstet Gynecol.* 2019;133:e377–84.
16. Lunenfeld B, Bilger W, Longobardi S, et al. The development of gonadotropins for clinical use in the treatment of infertility. *Front Endocrinol.* 2019;10:429. doi: [10.3389/fendo.2019.00429](https://doi.org/10.3389/fendo.2019.00429).
17. Mol BW, Bossuyt PM, Sunkara SK, et al. Personalized ovarian stimulation for assisted reproductive technology: Study design considerations to move from hype to added value for patients. *Fertil Steril.* 2018;109:968–79. doi: [10.1016/j.fertnstert.2018.04.037](https://doi.org/10.1016/j.fertnstert.2018.04.037).
18. Orvieto R. Triggering final follicular maturation-hCG, GnRH-agonist or both, when and to whom? *J Ovar Res.* 2015;8:60. doi: [10.1186/s13048-015-0187-6](https://doi.org/10.1186/s13048-015-0187-6).
19. Practice Committee of the American Society for Reproductive Medicine. Diagnostic evaluation of the infertile female: A committee opinion. *Fertil Steril.* 2015;103:e44–50. <https://doi.org/10.1016/j.fertnstert.2015.03.019>.
20. The ESHRE Guideline Group on Ovarian Stimulation, Bosch E, Broer S, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI. *Hum Reprod Open.* 2020;2020:hoaa009. doi: [10.1093/hropen/hoaa009](https://doi.org/10.1093/hropen/hoaa009).
21. Fatemi H, Bilger W, Denis D, et al. Dose adjustment of follicle-stimulating hormone (FSH) during ovarian stimulation as part of medically-assisted reproduction in clinical studies: A systematic review covering 10 years (2007–2017). *Reprod Biol Endocrinol.* 2021;19:68. doi: [10.1186/s12958-021-00744-x](https://doi.org/10.1186/s12958-021-00744-x).
22. Mahony MC, Hayward B, Mottla GL, et al. Recombinant human follicle-stimulating hormone alfa dose adjustment in US clinical practice: An observational, retrospective analysis of a real-world electronic medical records database. *Front Endocrinol.* 2021;12:742089. doi: [10.3389/fendo.2021.742089](https://doi.org/10.3389/fendo.2021.742089).
23. Patrizio P, Vaiarelli A, Levi Setti PE, et al. How to define, diagnose and treat poor responders? Responses from a worldwide survey of IVF clinics. *Reprod Biomed Online.* 2015;30:581–92. doi: [10.1016/j.rbmo.2015.03.002](https://doi.org/10.1016/j.rbmo.2015.03.002).
24. Yovich J. Monitoring the Stimulated IVF Cycle. pp. 94–120, 2019. <https://www.cambridge.org/core/books/abs/how-to-prepare-the-egg-and-embryo-to-maximize-ivf-success/monitoring-the-stimulated-ivf-cycle/3128E415EB0AB82EEDF98DEF155B124>.
25. Sachs Guedj N, Hart R, Requena A, et al. P-641 Physicians' practices towards hormone monitoring during ovarian stimulation in context of treatment with assisted reproductive technology (ART). A global survey conducted through IVFworldwide.com. *Hum Reprod.* 2022;37:deac107.590.
26. Farquhar C, Marjoribanks J, Brown J, et al. Management of ovarian stimulation for IVF: Narrative review of evidence provided for world health organization guidance. *Reprod Biomed Online.* 2017;35:3–16. doi: [10.1016/j.rbmo.2017.03.024](https://doi.org/10.1016/j.rbmo.2017.03.024).
27. Practice Committee of the American Society for Reproductive Medicine. Prevention and treatment of moderate and severe ovarian hyperstimulation syndrome: A guideline. *Fertil Steril.* 2016;106:1634–47. doi: [10.1016/j.fertnstert.2016.08.048](https://doi.org/10.1016/j.fertnstert.2016.08.048).
28. Vermeulen N, Le Clef N, D'Angelo A, et al. Manual for development of good practice recommendations. 2019. <https://www.eshr.eu/Guidelines-and-Legal/Guidelines/Guideline-development-process> (accessed September 2020).
29. Kaponis A, Chronopoulou E, Decavalas G. The curious case of premature luteinization. *J Assist Reprod Genet.* 2018;35:1723–40. doi: [10.1007/s10815-018-1264-8](https://doi.org/10.1007/s10815-018-1264-8).
30. Wang M, Xi Q, Yang Q, et al. The relationship between a novel evaluation parameter of premature luteinization and IVF outcomes. *Reprod Biomed Online.* 2021;42:323–31. doi: [10.1016/j.rbmo.2020.10.009](https://doi.org/10.1016/j.rbmo.2020.10.009).
31. Hart R, D'Hooghe T, Dancet E, et al. Self-monitoring of urinary hormones in combination with telemedicine - a timely review and opinion piece in medically assisted reproduction. *Reprod Sci.* 2021;1–14. doi: [10.1007/s43032-021-00754-5](https://doi.org/10.1007/s43032-021-00754-5).
32. International Federation of Fertility Societies. Standard 19: Clomiphene and its use in ovulation induction - Guidance for practitioners. 2017. <https://www.iffsreproduction.org/our-journal/practice-standards/> (accessed September 2020).
33. Brod M, Fennema H. Validation of the controlled ovarian stimulation impact measure (COSI): Assessing the patient perspective. *Health Qual Life Outcomes.* 2013;11:130. doi: [10.1186/1477-7525-11-130](https://doi.org/10.1186/1477-7525-11-130).
34. Dancet EA, Nelen WL, Sermeus W, et al. The patients' perspective on fertility care: A systematic review. *Hum Reprod Update.* 2010;16:467–87. doi: [10.1093/humupd/dmq004](https://doi.org/10.1093/humupd/dmq004).
35. Dancet EA, Van Empel IW, Rober P, et al. Patient-centred infertility care: A qualitative study to listen to the patient's voice. *Hum Reprod.* 2011;26:827–33. doi: [10.1093/humrep/der022](https://doi.org/10.1093/humrep/der022).
36. Dahhan T, van der Veen F, Bos AME, et al. The experiences of women with breast cancer who undergo fertility preservation. *Hum Reprod Open.* 2021;2021:hoab018. doi: [10.1093/hropen/hoab018](https://doi.org/10.1093/hropen/hoab018).
37. Redshaw M, Hockley C, Davidson LL. A qualitative study of the experience of treatment for infertility among women who successfully became pregnant. *Hum Reprod.* 2007;22:295–304. doi: [10.1093/humrep/del344](https://doi.org/10.1093/humrep/del344).
38. Lankreijer K, D'Hooghe T, Sermeus W, et al. Development and validation of the FertiMed questionnaire assessing patients' experiences with hormonal fertility medication. *Hum Reprod.* 2016;31:1799–808. doi: [10.1093/humrep/dew111](https://doi.org/10.1093/humrep/dew111).

39. van den Wijngaard L, Rodijk IC, van der Veen F, et al. Patient preference for a long-acting recombinant FSH product in ovarian hyperstimulation in IVF: A discrete choice experiment. *Hum Reprod.* 2015;30:331–7. doi: [10.1093/humrep/deu307](https://doi.org/10.1093/humrep/deu307).
40. Boedt T, Matthys C, Lie Fong S, et al. Systematic development of a mobile preconception lifestyle programme for couples undergoing IVF: The PreLiFe-programme. *Hum Reprod.* 2021;36:2493–505. doi: [10.1093/humrep/deab166](https://doi.org/10.1093/humrep/deab166).
41. Duthie EA, Cooper A, Davis JB, et al. A conceptual framework for patient-centered fertility treatment. *Reprod Health.* 2017;14:114. doi: [10.1186/s12978-017-0375-5](https://doi.org/10.1186/s12978-017-0375-5).
42. Shandley LM, Hipp HS, Anderson-Bialis J, et al. Patient-centered care: Factors associated with reporting a positive experience at United States fertility clinics. *Fertil Steril.* 2020;113:797–810. doi: [10.1016/j.fertnstert.2019.12.040](https://doi.org/10.1016/j.fertnstert.2019.12.040).
43. Huppelschoten AG, de Bruin JP, Kremer JA. Independent and web-based advice for infertile patients using fertility consult: Pilot study. *JMIR Formative Research.* 2019;3:e13916. doi: [10.2196/13916](https://doi.org/10.2196/13916).
44. Gerris J, Geril A, De Sutter P. Patient acceptance of self-operated endovaginal telemonitoring (SOET): Proof of concept. *Fact View Vis Obstyn.* 2009;1:161–70.
45. Gerris J, De Sutter P. Self-operated endovaginal telemonitoring (SOET): A step towards more patient-centred ART? *Hum Reprod.* 2010;25:562–8. doi: [10.1093/humrep/dep440](https://doi.org/10.1093/humrep/dep440).
46. World Health Organization. Task force on methods for the determination of the fertile period, special programme of research, development and research training in human reproduction. Temporal relationships between indices of the fertile period. *Fertil Steril.* 1983;39:647–55. doi: [10.1016/s0015-0282\(16\)47060-3](https://doi.org/10.1016/s0015-0282(16)47060-3).
47. Blackwell LF, Cooke DG, Brown S. The use of estrone-3-glucuronide and pregnanediol-3-glucuronide excretion rates to navigate the continuum of ovarian activity. *Front Pub Health.* 2018;6:153. doi: [10.3389/fpubh.2018.00153](https://doi.org/10.3389/fpubh.2018.00153).
48. Rapi S, Fuzzi B, Mannelli M, et al. Estrone 3-glucuronide chemiluminescence immunoassay (LIA) and 17beta estradiol radioimmunoassay (RIA) in the monitoring of superovulation for in vitro fertilization (IVF): Correlation with follicular parameters and oocyte maturity. *Acta Europaea Fertilitatis.* 1992;23: 63–8.
49. Severy LJ, Robinson J, Findley-Klein C, et al. Acceptability of a home monitor used to aid in conception: Psychosocial factors and couple dynamics. *Contraception.* 2006;73:65–71. doi: [10.1016/j.contraception.2005.07.008](https://doi.org/10.1016/j.contraception.2005.07.008).
50. Zaat TR, de Bruin JP, Goddijn M, et al. Home- or hospital-based monitoring to time frozen embryo transfer in the natural cycle? Patient-reported outcomes and experiences from the Antarctica-2 randomised controlled trial. *Hum Reprod.* 2020;35:866–75. doi: [10.1093/humrep/deaa040](https://doi.org/10.1093/humrep/deaa040).
51. Behre HM, Kuhlage J, Gassner C, et al. Prediction of ovulation by urinary hormone measurements with the home use ClearPlan fertility monitor: Comparison with transvaginal ultrasound scans and serum hormone measurements. *Hum Reprod.* 2000;15:2478–82. doi: [10.1093/humrep/15.12.2478](https://doi.org/10.1093/humrep/15.12.2478).
52. Shochet T, Comstock IA, Ngoc NTN, et al. Results of a pilot study in the U.S. and Vietnam to assess the utility And acceptability of a multi-level pregnancy test (MLPT) for home monitoring of hCG trends after assisted reproduction. *BMC Women's Health.* 2017;17:67. doi: [10.1186/s12905-017-0422-y](https://doi.org/10.1186/s12905-017-0422-y).
53. Fiers T, Dielen C, Somers S, et al. Salivary estradiol as a surrogate marker for serum estradiol in assisted reproduction treatment. *Clin Biochem.* 2017;50:145–9. <https://doi.org/10.1016/j.clinbiochem.2016.09.016>.
54. Ekeland AG, Bowes A, Flottorp S. Effectiveness of telemedicine: A systematic review of reviews. *Int J Med Inform.* 2010;79:736–71. doi: [10.1016/j.ijmedinf.2010.08.006](https://doi.org/10.1016/j.ijmedinf.2010.08.006).
55. Diaz VA, Player MS. Direct-to-patient telehealth: Opportunities and challenges. *Rhode Island Med J.* 2020;103:35–7.
56. Yokomizo R, Nakamura A, Sato M, et al. Smartphone application improves fertility treatment-related literacy in a large-scale virtual randomized controlled trial in Japan. *NPJ Digit Med.* 2021;4:163. doi: [10.1038/s41746-021-00530-4](https://doi.org/10.1038/s41746-021-00530-4).
57. Turner AP, Roubinov DS, Atkins DC, et al. Predicting medication adherence in multiple sclerosis using telephone-based home monitoring. *Disabil Health J.* 2016;9:83–9. doi: [10.1016/j.dhjo.2015.08.008](https://doi.org/10.1016/j.dhjo.2015.08.008).
58. Brichetto G, Pedulla L, Podda J, et al. Beyond center-based testing: Understanding and improving functioning with wearable technology in MS. *Mult Scler.* 2019;25:1402–11. 2019/09/11. doi: [10.1177/1352458519857075](https://doi.org/10.1177/1352458519857075).
59. Buyse GM, Rummey C, Meier T, et al. Home-based monitoring of pulmonary function in patients with duchenne muscular dystrophy. *Journal Neuromuscul Dis.* 2018;5:419–30. doi: [10.3233/jnd-180338](https://doi.org/10.3233/jnd-180338).
60. Carlson AL, Mullen DM, Bergenstal RM. Clinical use of continuous glucose monitoring in adults with type 2 diabetes. *Diabetes Technol Ther.* 2017;19:S4–S11. doi: [10.1089/dia.2017.0024](https://doi.org/10.1089/dia.2017.0024).
61. Liyanage-Don N, Fung D, Phillips E, et al. Implementing home blood pressure monitoring into clinical practice. *Curr Hypertens Rep.* 2019;21:14. doi: [10.1007/s11906-019-0916-0](https://doi.org/10.1007/s11906-019-0916-0).
62. Nastri CO, Ferriani RA, Rocha IA, et al. Ovarian hyperstimulation syndrome: Pathophysiology and prevention. *J Assist Reprod Genet.* 2010;27:121–8. doi: [10.1007/s10815-010-9387-6](https://doi.org/10.1007/s10815-010-9387-6).
63. Meyer L, Murphy LA, Gumer A, et al. Risk factors for a suboptimal response to gonadotropin-releasing hormone agonist trigger during in vitro fertilization cycles. *Fertil Steril.* 2015;104:637–42. doi: [10.1016/j.fertnstert.2015.06.011](https://doi.org/10.1016/j.fertnstert.2015.06.011).
64. Mesen TB, Young SL. Progesterone and the luteal phase: A requisite to reproduction. *Obstet Gynecol Clin North Am.* 2015;42:135–51. doi: [10.1016/j.jogc.2014.10.003](https://doi.org/10.1016/j.jogc.2014.10.003).
65. Magini A, Pinzani P, Bolelli GF, et al. Measurement of estrone-3-glucuronide and pregnanediol-3 alpha-glucuronide in early morning urine samples to monitor ovarian function. *J Biolumin Chemilumin.* 1989;4:567–74. doi: [10.1002/bio.1170040174](https://doi.org/10.1002/bio.1170040174).
66. Pazzagli M, Magini A, Forti G, et al. Measurement of glucuronometabolites of 17 beta-estradiol and progesterone in diluted overnight urine. An approach to the study of luteal insufficiency. *J Steroid Biochem.* 1987;27:399–404. doi: [10.1016/0022-4731\(87\)90333-5](https://doi.org/10.1016/0022-4731(87)90333-5).
67. Allende ME, Arraztoa JA, Guajardo U, et al. Towards the clinical evaluation of the luteal phase in fertile women: A preliminary study of normative urinary hormone profiles. *Front Public Health.* 2018;6:147. doi: [10.3389/fpubh.2018.00147](https://doi.org/10.3389/fpubh.2018.00147).
68. Valdera Simbrón CJ, Hernández Rodríguez C. Telemedicine in assisted reproduction. *Reprod Biomed Online.* 2021;42:269–72. doi: [10.1016/j.rbmo.2020.11.016](https://doi.org/10.1016/j.rbmo.2020.11.016).
69. Hernández C, Valdera CJ, Cordero J, et al. Impact of telemedicine on assisted reproduction treatment in the public health system. *J Healthcare Qual Res.* 2020;35:27–34. doi: [10.1016/j.jhqr.2019.08.004](https://doi.org/10.1016/j.jhqr.2019.08.004).
70. Veiga A, Gianaroli L, Ory S, et al. Assisted reproduction and COVID-19: A joint statement of ASRM, ESHRE and IFFS. *Hum Reprod Open.* 2020;3:hoa033.
71. German IVF Register D.I.R. D.I.R special evaluation: Effects of the COVID-19 situation on reproductive medicine in Germany, 2020 as a whole. 2021. Available from <https://www.deutsches-ivf-register.de/aktuelle-nachrichten-des-dir.php> (Accessed March 2021).
72. Smith ADAC, Gromski PS, Rashid KA, et al. Population implications of cessation of IVF during the COVID-19 pandemic. *Reprod Biomed Online.* 2020;41:428–30. doi: [10.1016/j.rbmo.2020.07.002](https://doi.org/10.1016/j.rbmo.2020.07.002).

73. Esposito V, Rania E, Lico D, et al. Influence of COVID-19 pandemic on the psychological status of infertile couples. *Eur J Obstet Gynecol Reprod Biol.* 2020;253:148–53. doi: [10.1016/j.ejogrb.2020.08.025](https://doi.org/10.1016/j.ejogrb.2020.08.025).
74. Vaughan DA, Shah JS, Penzias AS, et al. Infertility remains a top stressor despite the COVID-19 pandemic. *Reprod Biomed Online.* 2020;41:425–7. doi: [10.1016/j.rbmo.2020.05.015](https://doi.org/10.1016/j.rbmo.2020.05.015).
75. World Health Organization. Maintaining essential health services: operational guidance for the COVID-19 context. Interim guidance 1 June 2020. 2020. <https://www.who.int/teams/sexual-and-reproductive-health-and-research/key-areas-of-work/sexual-reproductive-health-and-rights-in-health-emergencies/covid-19> (accessed 26 November 2020).
76. World Health Organization. Infertility definitions and terminology. 2021. <https://www.who.int/teams/sexual-and-reproductive-health-and-research/key-areas-of-work/fertility-care/infertility-definitions-and-terminology> (accessed February 2021).
77. Vaughan DA, Yin SH, Shah JS, et al. Telemedicine for reproductive medicine: Pandemic and beyond. *J Assist Reprod Genet.* 2022;39:327–9. doi: [10.1007/s10815-021-02383-y](https://doi.org/10.1007/s10815-021-02383-y).

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OOCYTE COLLECTION

Andrew Murray and Gab Kovacs

History

The very first human pregnancy using *in vitro* fertilization (IVF) was achieved using laparotomy for obtaining the oocyte [1]. Meanwhile, Morgenstern and Soupart [2] in 1972 had described an experimental procedure for both abdominal and vaginal approaches to oocyte recovery, using a special oocyte recovery unit, in conjunction with gynaecological surgery. As laparotomy was very invasive and laparoscopy was just beginning to be applied to gynaecology, the laparoscopic approach for oocyte collection became routine by the late 1970s [3, 4].

It was the expertise of Patrick Steptoe with laparoscopy that resulted in his successful partnership with Robert Edwards, resulting in the birth of Louise Brown in 1978. It was the laparoscopic approach with modification of the collection needle [5] that was used in the stimulated/controlled cycles that resulted in the next nine births from the Monash team, which converted IVF from a research tool to clinical treatment. Laparoscopy was also used by the Jones's team when they used human menopausal gonadotropins to achieve the first pregnancies in the United States [6].

During the early 1980s, IVF became available worldwide, using laparoscopic oocyte collection. It was the pioneering work of Susan Lenz in Copenhagen [7] and Wilfred Feichtinger [8] in Vienna that changed oocyte collection from laparoscopic to the far less-invasive transvaginal ultrasound-guided technique. With its efficacy being proven to be as good as laparoscopy by a comparative study by Kovacs and colleagues [9], most of the world's IVF units abandoned laparoscopy for the transvaginal route.

Anaesthesia/analgesia

The shift towards ultrasound-guided oocyte collection has enabled clinics to offer simpler, outpatient-based anaesthesia and analgesia. Many clinics still perform oocyte collection under general anaesthetic, however the majority of clinics throughout the world now use intravenous sedation. Monitored anaesthesia is relatively easy to deliver, drugs are well tolerated and best suited in day care settings. In the United States, 95% of the programs use conscious sedation as a part of monitored anaesthesia care [10]. In the United Kingdom, 84% of the centres now use sedation [11].

Vlahos and colleagues in 2009 [12] undertook a survey that found that conscious sedation was the most popular method used. It has a relatively low risk of adverse events and no effects on oocyte and embryo quality or pregnancy rates. In 2013, Kwan and colleagues [13] carried out a Cochrane analysis to assess the effectiveness and safety of different methods of conscious sedation and analgesia on pain relief and pregnancy outcomes in women undergoing transvaginal oocyte retrieval. They compared randomized controlled trials comparing different methods of conscious sedation and analgesia for pain relief during oocyte recovery using various adjuncts such as para-cervical block,

acupuncture, and various analgesic agents. They analysed a total of 21 trials including 2974 women undergoing oocyte retrieval. Unfortunately, there was inconsistency between the trials and small numbers of cases reported, so it is no surprise that conflicting results were found. Their findings did not support the superiority of one particular method or technique over another. All the approaches appeared to be acceptable and were associated with a high degree of satisfaction in women. As women vary in their experience of pain and in coping strategies, the optimal method may be individualized depending on the preferences of both the women and the clinicians, as well as resource availability.

In 2019, Guasch and colleagues [14] conducted a review of anaesthesia and analgesia for transvaginal oocyte retrieval. They reached similar conclusions to the Cochrane team, that is no technique was superior, and that the evidence available at that time was not convincing enough to recommend or avoid any anaesthetic technique in terms of pregnancy and birth rates.

Conscious sedation with the concomitant use of local anaesthesia is undoubtedly less expensive than general anaesthesia and does not require the presence of an anaesthetist. Furthermore, for ambulatory procedures, conscious sedation is preferable for the patients, as their recovery times are shorter in comparison with general anaesthesia [15].

Cleansing/sterilizing the vagina

When the transvaginal route of oocyte collection was introduced in the mid-1980s, there was concern that entering the peritoneal cavity through a potentially infected field (the vagina) may result in pelvic infection. There were therefore attempts to carry out routine preoperative sterilization with antiseptic solutions. This then resulted in anxiety that the antiseptic may be toxic to the oocytes collected. Van Os and colleagues [16] carried out a prospective randomized study that showed that using 1% povidone iodine and normal saline washout resulted in a lower pregnancy rate (30.3% vs 17.2%) and therefore was not advisable. They simultaneously showed that there was no significant infection risk in the saline group, as it had no higher incidence of infection than the iodine group. Hannoun and colleagues [17] also studied whether washing out the vagina with saline after preparation by iodine affected outcomes. They found not washing out was associated with an increase in the rate of chemical pregnancy, and they recommended that it is advisable to cleanse after iodine before oocyte aspiration. Supportive evidence comes from a more recent study from Osaka, Japan [18]. These authors compared 956 infertile patients undergoing vaginal preparation with saline douching alone versus 1216 infertile patients undergoing a combination of povidone iodine disinfection and subsequent saline douching in an IVF program. They recorded four infections in the saline douching-alone group and none in the combination group, which was a statistically significant difference ($p = 0.016$). There were no significant differences in the rate of fertilization, morphologically

good embryo development, and clinical and ongoing pregnancy rates between the two groups. They advocated the use of vaginal povidone iodine disinfection and subsequent saline douching to prevent infection, and concluded that the regime had no evidence of harming oocyte quality.

Today, there is still no consensus on what vaginal preparation is optimal. Many surgeons carry out no vaginal preparation and simply insert the needle through the vagina. Others wash out with saline, while some use betadine followed by saline lavage. The experience at Monash IVF with no vaginal preparation of over 100,000 transvaginal oocyte collections is that post-operative infection is rare, unless an endometrioma has been entered. Younis and colleagues [19] reported as early as 1997 that severe endometriosis with ovarian endometriomata seems to be a significant risk factor for pelvic abscess development following transvaginal oocyte pickup for IVF embryo transfer. They proposed that the presence of old blood in an endometrioma provides a culture medium in which bacteria can grow after transvaginal inoculation. If an endometrioma is encountered during oocyte collection, prophylactic antibiotics are recommended. This is also good practice for patients with a history of pelvic inflammatory disease (PID), pelvic adhesions, dermoids, or previous pelvic surgery who may be deemed at higher risk of pelvic infection. There is no evidence for the use of antibiotic prophylaxis in low-risk patients.

The equipment

The suction source

In the early days, manual suction was conducted using a needle, plastic tubing, and syringe [2]. Berger and colleagues devised a special aspiration unit, with a 20-gauge, 10-inch needle connected by a polyethylene tube to a 10-mm Vacutainer, which then connected to a vacuum bottle with an adjustable pressure gauge. The suction was turned on or off by a thumb valve. The technique then was modified with the use of a suction pump operated by a foot pump [5]. Today, sophisticated suction pumps with adjustable aspiration pressures are widely available commercially.

The suction

There has been surprisingly little study undertaken on the physical aspects of oocyte recovery. A study by the team at Monash published the findings of experiments on bovine eggs carried out in the laboratories of Cook Medical Technology in Brisbane, Australia [20]. Some of the observations of these studies are outlined later. In this study, the velocity and flow rates of oocytes through the collection system were measured, and the damaging effect of non-laminar flow to the oocyte was observed.

Application of vacuum to the follicle

Vacuum applied after needle entry into the follicle

After application of the vacuum, the pressure within the system equilibrates, resulting in a steady flow rate until the fluid volume decreases and the follicle collapses, so that the follicular wall blocks the lumen of the needle. The time for the system to equilibrate depended on the vacuum pressure, the diameter of the needle, and the volume of the follicle. Maximum flow was achieved when the pressure was at a steady state. Should air be sucked into the system by entering around where the needle pierced the follicle wall, frothing with non-laminar flow resulted, the so-called cappuccino effect. This has a deleterious effect on the oocyte, as it is thrown around the collection system.

Vacuum deactivated before the needle was withdrawn from the follicle

If the pressure was deactivated whilst the needle was still in the follicle (and there were no leaks), the pressure within the needle and collecting tube drops, and there is often backflow towards the follicle. This can result in the oocyte being sucked back and possibly lost. The amount of backflow depends on how much air enters the system and how much higher the collection tube is above the patient's pelvis.

The vacuum profiles within the aspiration system

It was estimated that when using the system at 150 kPa it took five seconds for the system to stabilize. The pressure within the follicle before penetration varies depending on the size (maturity), shape, and position of the follicle. The internal pressure increases correlating with size. However, due to the pressure caused by the needle deforming the surface of the follicle at the time of puncture, the pressure within the follicle may be much higher (up to 60 mmHg). The blunter the needle, the higher the resultant pressure. This may result in follicular fluid being lost as it spurts out during this process. If the pressure is already applied, some/most of this fluid will be aspirated as it escapes along the outer wall of the follicle.

There is a pressure gradient down the collection system, so that the pressure at the tip of the needle is only 5% of the pressure at the pump. The oocyte is therefore exposed to ever-increasing pressures as it travels along the needle, the collection tube, and the collecting test tube. Excessive pressure can cause the ovum to swell and the zona to crack.

Follicle and needle volumes

Table 50.1 lists the respective volumes contained in follicles between 6 and 20 mm in diameter. A 6-mm follicle only contains 0.1 mL, so that 10–12 follicles need to be emptied before the “dead space” of 1.0–1.2 mL in a standard needle and collecting tube is filled and fluid reaches the collection test tube.

Application of the vacuum

Following the penetration of the follicle by the needle and the application of suction, the pressure within the follicle, the needle,

TABLE 50.1 The Diameter to Volume Ratios of Typical Follicles

Follicle Diameter (mm)	Follicle Volume (mL)
6	0.1
7	0.2
8	0.3
9	0.4
10	0.5
11	0.7
12	0.9
13	1.1
14	1.4
15	1.8
16	2.1
17	2.6
18	3.0
19	3.6
20	4.2

Note: The typical dead spaces of needles and collecting tubules are 1.0–1.2 mL.

and the collecting tube equilibrates. If there is a tight seal around the needle (i.e. the needle was sharp and was introduced precisely through the follicular wall and the hand is kept still so that tearing does not result), when the suction pressure is reduced, there will be backflow of fluid into the follicle. This can result in the oocyte being lost. On the other hand, if the needle is withdrawn whilst the suction is still being applied, there is a sudden change of pressure at the needle tip from the high vacuum of the follicle to atmospheric pressure, with a rapid surge of fluid towards the collection tube. If the oocyte is contained in the terminal portion of the fluid, it is subjected to increased speeds of travel as well as turbulence, resulting in loss of the cumulus mass and even fracture of the zona pellucida.

Damage within the follicle

During aspiration, the oocyte has to accelerate from a resting state to the velocity of the fluid within the needle. If this is too rapid, the cumulus may be stripped off. The higher the aspiration pressure, the greater the risk, and the smaller the follicle, the higher the pressure that is needed. This may be particularly relevant in the collection of immature oocytes for *in vitro* maturation.

Damage to oocytes

It was noted that high velocities of flow may strip the cumulus from the oocyte. Even with laminar flow, there are significant differences in velocity of the follicular fluid within the centre of the needle compared to the periphery. This can result in "drag" on the outer layers of the cumulus, resulting in potential damage. The longer the needle, the smaller its internal diameter, and the greater the pressure required to maintain the same velocity. It was found that when a 17-gauge collection needle was used, all oocytes lost their cumulus mass when the aspiration pressure reached 20 kPa (150 mmHg). It is therefore recommended that pressures be kept below 120 mmHg.

Apart from the speed of travel, turbulent non-laminar flow can also damage the oocyte, either stripping its cumulus mass or fracturing the zona. It is believed that an intact cumulus may be important in preventing damage to oocytes.

The needle

The initial aspiration system consisted of a single-lumen needle. This had to be disconnected at the hub from the suction tubing if follicular flushing was required. There was also always a dead space of 1.0–1.2 mL, and the oocyte would often be flushed up and down in the collection system. It would only be finally recovered when the needle was removed and flushed with fluid—the "needle wash." To allow simpler irrigation of the follicle "flushing," the concept of a double-channel needle was introduced. This required a channel used for oocyte aspiration, with a side channel where fluid could be injected into the follicle. It also allowed simultaneous flushing and aspiration. Scott and colleagues [21] compared single- and double-lumen needle aspirations, albeit with only 22 patients in each arm. Although there were no differences between the two needles in the number of oocytes provided for IVF, there were technical differences. The double-lumen needle was more flexible and frequently deviated from the projected path as observed by ultrasound. The single-lumen needle may be preferable because it is technically easier to use. Haydardedeoglu and colleagues [22] compared the retrieval efficiency of the single-lumen technique (only aspirating) with a double-lumen approach where flushing was also used. They found that there was no improvement in outcome with respect to oocyte numbers, clinical pregnancy rate, or live birth rate.

A unique quasi-double-lumen needle has been designed by Steiner [23]. The needle is composed of three parts: a 7-cm-long, 21-gauge needle that penetrates the vagina and ovary; an adjacent, rigid, 17-gauge tube that carries aspirates and flush media back to a collection tube; and a plastic sheath surrounding this tube for carrying flush media, which connects to a flush syringe on one end and extends down to the top of the 21-gauge needle on the other end. There are holes drilled in the 21-gauge needle so that flush media goes through it into the ovary. The suction on the longer 17-gauge tube pulls the aspirate and flush media away from the patient and into the media collection tube. A major difference between this needle and a standard 19-gauge single-lumen needle is the amount of dead space. The dead space in a 19-gauge needle will hold the fluid contained in more than four 6-mm follicles, whereas the dead space in the Steiner needle will hold the fluid from only one follicle. This enables flushing with a single lumen needle, with minimal time added to the procedure.

Does size matter?

The original Teflon-lined needle devised at Monash IVF in 1980 [5] was a 19-gauge needle. Attempts have been made to compare different needle diameters. A prospective comparative study by Kushnir and colleagues [24] in 2013, where they used a 17-gauge needle for one ovary and a 20-gauge needle for the other, concluded that needle diameter did not affect oocyte yield, yet the smaller-diameter needle prolonged the operative time. Currently, the most commonly used needle in Australasia is a 16-gauge needle of 300 mm length.

In a study of 47 women, Buisman and colleagues [25] concluded that a finer (20/17-gauge) aspiration needle resulted in less pain during and after the oocyte collection.

Atzmon et al. [26] compared two different needles used for oocyte pickup (OPU) to assess whether the different stress forces along the needle affect the presence of degenerative oocytes, oocyte quality, and embryo morphokinetics. These researchers carried out a prospective randomized study with the embryologist blinded, where they compared 17-gauge and 20/17-gauge needles. They studied 43 women from whom 580 oocytes were collected, 293 with a 17-gauge aspiration needle and 287 with a 20/17-gauge aspiration needle.

Oocytes were called abnormal if any of the following parameters were abnormal: polar body shape, zona pellucida, cytoplasm, perivitelline space, or vacuoles.

Oocyte scoring: only mature meiotic metaphase II (MII) oocytes were analysed. Based on the five standard parameters for oocyte quality described by Rienzi et al. and by Balaban et al. [11], each oocyte was evaluated according to (i) size and symmetry of the perivitelline space structure, (ii) colour and integrity of the cytoplasm, (iii) intactness of zona pellucida, (iv) polar body morphology, and (v) presence of vacuoles. Each parameter was given a value of 0 if normal and -1 if an abnormality was present. All negative parameters were summed. Scores could range from 0 to -5. A total score of 0 was considered best oocyte quality. They found that on oocyte scoring, on embryo quality, and pregnancy rate the results were comparable between the two needles used.

Technique

Flushing or rapid oocyte collection

When transvaginal oocyte collection was first undertaken, the technique of laparoscopic harvesting was transferred to the transvaginal approach. Follicles were initially aspirated, and then repeatedly flushed to try and recover as many oocytes as possible.

This, however, is time-consuming and also uses large quantities of culture medium. It was soon recognized that most oocytes can be recovered by just aspirating, and that the follicular fluid from the next follicle will often flush the oocyte into the collection tube. This was called the rapid oocyte recovery technique. Hill and Levens [27] reviewed the evidence regarding the effectiveness of ovarian follicular flushing in improving oocyte yield in 2010. They concluded that follicular flushing offers no substantive benefit to oocyte yield, fertilization rates, or pregnancy outcomes for normal and poor-responding patients. When undertaking natural cycle or minimal stimulation, follicular flushing may result in more mature embryos.

Wongtra-Ngan and colleagues [28] in Thailand undertook a Cochrane review of studies comparing flushing to simple aspiration. They found no difference in oocyte numbers, or other clinical outcomes, but did find that operative time was significantly increased (3–15 minutes) by flushing. In contrast, a small trial from France [29] in women undergoing minimal stimulation found that flushing in this group resulted in better embryo morphology and implantation rates, but not increased clinical pregnancy rates.

For many years now at Fertility Associates, New Zealand's largest provider of IVF, double lumen needles and flushing have been abandoned, even for those women with three or fewer follicles. There has been no increased rate of procedures where no oocytes were recovered.

A Cochrane review [30] has looked at the issue of aspiration alone versus flushing at transvaginal ultrasound-guided oocyte collection. Ten studies containing 928 women were reported in the randomized controlled trials (RCTs) included in the analysis. There was no difference in any parameters, in particular live birth rate (LBR) in aspiration only were 41% for aspiration only and 29%–52% if additional flushing used. There was no difference in the clinical pregnancy rates, number of oocytes collected, and no difference in number of embryos available for transfer nor freezing.

One group of patients, for whom many clinicians still flush, are those with mono-follicular development. Schwartz and colleagues [31] report a prospective randomized trial comparing aspirating and flushing against flushing only. They studied 164 mono-follicular oocyte collections, with aspiration carried out in 83. They obtained significantly more oocytes ($p = 0.02$) with flushing (77.1%) than aspiration alone (59.3%). They reported that most oocytes with flushing were obtained within the first three flushes.

Fertilization rate was also higher in the flushing group—63.9% against 46.9% in the aspiration only group—which was just significant ($P = 0.45$). However, the final outcome, either as clinical pregnancy rate or live birth rate, found no significant difference between the groups. Nevertheless, the authors concluded that their study proved that flushing of single follicles in mono-follicular IVF increases the oocyte yield.

Contrasting with this was a study by Calabre and colleagues [32] who reported another RCT of aspiration with flushing against aspiration only in “poor responders” with four follicles. The overall mean oocyte numbers recovered were significantly higher in the “no flush” group (3.42) than in the “flush” group (2.41) ($p = 0.001$). However, once only metaphase II oocytes were considered, there was no significant difference (1.69 vs 2.07; $p = 0.148$). Concerning the secondary assessment criteria, there was no difference in terms of the number of transferable embryos (median one in both groups), fertilization rate (68.8% vs 75%), or live births (15 vs 13). In addition, the time taken for adding

aspiration increased from a mean of seven minutes in the “no flush” to 10 minutes in the “flush” group.

They concluded that follicular flushing in poor responders is not beneficial.

Curetting the follicle

In the early days of IVF using laparoscopy, each oocyte collection lasted an hour. Follicles were visualized directly, aspirated, flushed, and, if still no oocyte was collected, they were “curetted” with the needle [5]. With the change to ultrasound-guided oocyte collection, this practice has been abandoned. Nevertheless, Dahl and colleagues [33] retrospectively reviewed an unselected 275 cases of oocyte collection from 2003 to 2005 and concluded that patients undergoing follicle curetting had a 22% increase in oocyte yield, but not in live birth rates. This is not a practice that is widely used today.

Avoiding turbulent flow

When aspirating follicles, it is important to recognize that in order to fill the “dead space” between the needle tip and the aspiration tube, somewhere between 1 and 2 mL of follicular fluid is needed.

As already described, it is desirable to avoid damage to the cumulus–oocyte mass during aspiration. The aim is to avoid non-laminar flow within the collection tube, which is likely to damage the oocyte. Attention should be paid to filling the tubing with fluid prior to aspiration, using gentle changes in aspiration pressure, limiting the suction pressure, and stopping aspiration whilst withdrawing the needle to avoid the aspiration of air causing turbulence (the “cappuccino effect”).

Temperature control

Another important point is to deliver oocytes to the laboratory in the best condition, including minimizing the effect of cooling. Colleagues from New Zealand [34] investigated the effects of IVF aspiration on the temperature, pH, and dissolved oxygen of bovine follicular fluid. They found that the temperature of follicular fluid dropped by $7.7 \pm 1.3^\circ\text{C}$ upon aspiration. Dissolved oxygen levels rose by 5 ± 2 vol.%. The pH increased by 0.04 ± 0.01 , and the authors concluded that these changes could be detrimental to oocyte health, and, consequently, efforts should be made to minimize these changes. The collection tubes are therefore kept in a test tube warmer whilst they are waiting to be connected to the collection system. At the same group of clinics, it is now standard practice to “prime” the suction tubing with pre-warmed media, which minimizes these temperature and pH changes as well as removing the “dead space.”

The approach

It is important to have a systematic approach to performing oocyte retrieval. Table 50.2 outlines the recommended steps. After performing identity checks, checking for pre-existing medical problems, and administering sedation and analgesia, the procedure can commence. Priming the suction tubing with warmed culture media prior to commencement is an opportunity to check that the aspiration pressure is correct, and that the suction is working. The vaginal ultrasound probe is covered with a sterile cover and the sterile needle guide is attached. Ultrasound guides are bespoke for each brand of ultrasound probe, and will have been calibrated for accurate placement of the needle. It is good practice to image both ovaries to judge location, ease of access, and number of follicles. Generally, it is pragmatic to start with

TABLE 50.2 Oocyte Collection Checklist

- Check that operating list is in the correct order
- See patient in preadmission room
 - Check name, date of birth, and ID number
 - Check for any allergies (e.g. latex)
 - Check most recent ultrasound
 - Check most recent hormone levels (if performed)
 - Check consent form signed
 - Check whether any limit on the number of oocytes inseminated
 - Check whether it is standard *in vitro* fertilization or intracytoplasmic sperm injection
 - Check whether cleavage stage, blastocyst transfer, or “freeze-all”
- Check equipment
 - Ensure ultrasound machine works and check orientation of image
 - Check tubes connected to needle and suction pump (It is also good practice to have a backup machine in the case of pump failure.)
 - Test that suction is working and adjust pressure
 - Fill collection tube with warm media
- Procedure
 - Double-check that patient ID and names on collection tubes match—“time out”
 - Proceed with collection
 - Complete notes
 - Inform patient about the number of eggs collected

the ovary that is easiest to access first. If local anaesthetic is to be used, a single lumen 20G body, 17G tip 300mm long needle can be carefully placed through the vaginal mucosa, and then stopped just before it punctures the pelvic peritoneum. Care should be taken to avoid obvious vaginal wall vessels. Aspirating back and seeing no blood also ensures the needle tip is not in a vessel. Local anaesthetic (such as 0.25% Marcaine, 10 mL each side) can then be instilled, aiming to “tent” the peritoneum immediately beneath the ovary. With firm longitudinal pressure, both to immobilize the ovary and minimize vaginal bleeding, the ovary is punctured, suction applied, and each follicle drained. As each follicle drains, micro-movements with the probe are used to keep the tip of the needle in the centre of the follicle as it collapses. Just as the follicle collapses around the needle tip, rotate the needle between thumb and forefinger to “curette” the follicle. Ensure the follicles are fully drained before moving to the next follicle. Be careful not to mistake the iliac artery or vein for follicles—“beware of the pulsating follicle.” Economy of movement is the key; ideally follicles are drained sequentially along a longitudinal axis from proximal to distal end of the ovary as it relates to the vaginal probe. Maintain suction the entire time, but do not withdraw the needle into the vagina with suction deployed as this will aspirate bacteria into the system. After aspirating the first line of follicles, carefully withdraw the needle tip back towards the probe, but not into the vagina, adjust position, then move on to the next cohort. In most cases it should be possible to drain all follicles for each ovary from a single puncture on each side.

When changing from one ovary to the next, flush the needle with culture media to clear any clots.

At the conclusion of the procedure, a swab on a sponge holder can be applied to the vaginal fornices to provide pressure to the

puncture sites. The vagina should be inspected for any excessive bleeding and if there is none the procedure is concluded.

Complications

Transvaginal oocyte collection has become the method of choice during the last four decades. However, although complications are rare, several possible complications of transvaginal oocyte collection have been reported.

The most common operative complications are:

- Haemorrhage
- Trauma to pelvic structures
- Pelvic infection, tubo-ovarian, or pelvic abscess

Rarely reported complications include:

- Ovarian torsion
- Rupture of ovarian endometriosis
- Appendicitis
- Ureteral obstruction [35]
- Vertebral osteomyelitis [36]
- Anaesthetic complications

Data from ESHRE IVF Monitoring (EIM) on complications from OPU looked at 776,556 cycles, with complications reported in 1328 cycles (0.17%). These included 919 with bleeding (0.11%), 108 infections (0.013%), and 301 (0.038%) other complications related to oocyte retrieval [37].

Haemorrhage can result in vaginal bleeding at and after the oocyte collection (overt bleeding) or in intra-abdominal bleeding (covert bleeding). Bennet and colleagues [38] reported on a four-year prospective study carried out at King’s College, London, of 2670 consecutive procedures, reporting that vaginal haemorrhage occurred in 229 (8.6%) of the cases, with a significant loss (classified as more than 100 mL) in 22 cases (0.8%). Haemorrhage from the ovary with hemoperitoneum formation was seen on two occasions and necessitated emergency laparotomy in one instance. A single case of pelvic haematoma formation from a punctured iliac vessel was also recorded; this settled without intervention.

Nouri and colleagues [39] reviewed published series of cases of post-operative bleeding requiring surgical intervention, and noted that evidence of severe bleeding was obvious within one hour in a third of cases.

As early as the 1990s, it was recognized that pre-existing endometrioma was a risk factor for pelvic infection after oocyte collection. Younis and colleagues from Israel, in 1997 [19], reported on three infertile women with ovarian endometriomata who presented with late manifestation of severe pelvic abscess 40, 24, and 22 days after oocyte collection, respectively. Severe endometriosis with ovarian endometriomata seems to be a significant risk factor for pelvic abscess development. Late manifestation of pelvic abscess supports the notion that the presence of old blood in an endometrioma provides a culture medium for bacteria to grow after transvaginal inoculation. Moini and colleagues [40], working in Tehran, Iran, reported that during a six-year period, when 5958 transvaginal ultrasound-guided oocyte retrievals were carried out, 10 cases of acute pelvic inflammatory disease (0.12%) were observed. Eight of the 10 patients were diagnosed as infertile because of endometriosis. They concluded that this supports the previous reports that endometriosis can raise the risk of pelvic infection after oocyte retrieval. More vigorous antibiotic

prophylaxis and better vaginal preparation were recommended when oocyte pickup is performed in patients with endometriosis.

Overall, the risk of significant pelvic infection is between 1:200 and 1:500. Consequently, prophylactic antibiotics are not indicated, unless an endometrioma is entered or there is a past history of pelvic infection, and then it is our policy to administer a single dose of intravenous antibiotic (e.g. Cefuroxime).

Very uncommon complications

Ureteric obstruction

There is a case report from Greenville, SC [35], of acute ureteral obstruction following seemingly uncomplicated oocyte retrieval. Prompt diagnosis and ureteral stenting led to rapid patient recovery with no long-term urinary tract sequelae.

Jayakrishnan and colleagues [41] reported a case of pseudoaneurysm causing massive haematuria with hemodynamic instability occurring after oocyte retrieval. The patient required a blood transfusion, cystoscopy, and resection and cauterization of the pseudoaneurysm. They concluded that injury to surrounding structures should always be kept in mind during oocyte retrieval.

Vertebral osteomyelitis

The most bizarre complication reported after oocyte collection is vertebral osteomyelitis reported from Tel Aviv, Israel, by Almog and colleagues [36]. They reported a case of vertebral osteomyelitis as a complication of transvaginal oocyte retrieval in a 41-year-old woman who underwent IVF and embryo transfer treatment. After she returned with severe low back pain, vertebral osteomyelitis was diagnosed and treated with antibiotics.

Cullen's sign (periumbilical haematoma)

Bentov and colleagues [42] described two cases of periumbilical haematoma (Cullen's sign) following ultrasound-guided transvaginal oocyte retrieval. Spontaneous resolution of the symptoms occurred within two weeks. They concluded that the appearance of a periumbilical haematoma (Cullen's sign) following ultrasound-guided transvaginal oocyte retrieval reflects a retroperitoneal haematoma of a benign course.

Troubleshooting

It is important that before commencing oocyte collection the system is tested by aspirating some culture medium. This also provides a column of medium into which to collect the follicular fluid, eliminating dead space and thus encouraging laminar flow.

Should suction then subsequently decrease or stop, the following steps should be undertaken:

- Ensure that the suction pump is turned on and that the suction pedal is functioning (many aspiration pumps have a light that goes on, and some have audible signals when the pump is activated).
- Check that all connections of tubing between the aspiration tube and the pump are tightly connected.
- Exclude any cracks in the aspiration test tube.
- Ensure that the collection tubing is not kinked or damaged.
- Rotate the needle within the follicle to ensure that it is not blocked by follicular wall tissue.
- If there is still no suction, remove the needle and perform a "retrograde flush" to clear any blockage.
- Before re-inserting the needle, re-check by aspirating some culture medium.

Failure to get oocytes: Check that an appropriate trigger has been given

Sometimes several follicles are aspirated and no oocytes are recovered. If the fluid collected is very clear and devoid of cells (granulosa and cumulus), suspicion may be raised that the patient has not had her trigger hormone. If human chorionic gonadotropin (hCG) was used as the trigger, it is suggested that before follicles from the second ovary are aspirated, some of the follicular fluid is tested with a urinary pregnancy test strip. As these turn blue (react positive) when the concentration exceeds 25 mIU/mL of hCG, if it has been administered, there should be sufficient hCG in the follicle to give a positive result. If the test is negative, it is possible to abandon the collection, administer hCG, and defer the collection from the other ovary until about 36 hours later. Although the number of oocytes collected will be limited to one ovary, it is still possible to salvage the cycle.

Pre-treatment of pathology

It has long been suggested that tubal disease, and particularly hydrosalpinx, has a detrimental effect on the outcome of IVF. To determine whether surgical removal of hydrosalpinges improved outcome, Johnson and colleagues [43] undertook a Cochrane analysis of all trials comparing a surgical treatment for tubal disease with a control group generated by randomization. The studied outcomes were live birth (and ongoing pregnancy), pregnancy, ectopic pregnancy, miscarriage, multiple pregnancy, and complications. Three randomized controlled trials involving 295 couples were included in this review. The odds of ongoing pregnancy and live birth were increased with laparoscopic salpingectomy for hydrosalpinges prior to IVF. The odds of pregnancy were also increased, but there was no significant difference in the incidence of ectopic pregnancy. They recommended that laparoscopic salpingectomy should be considered for all women with hydrosalpinges prior to IVF treatment. They also concluded that the role of surgery for tubal disease in the absence of a hydrosalpinx is unclear and merits further evaluation.

Assessing clinical competence

It is recommended that prior to clinicians being credentialed for undertaking oocyte collections, a structured training program should be carried out. One approach is that the instructor aspires one side, and having collected some eggs, the trainee should do the other side under supervision. The number of supervised collections probably varies between 20 and 40 before the trainee should be allowed to perform collections on their own.

Ongoing assessment of clinical competence should then be regularly performed. Our clinical indicator is the oocyte collection rate: the number of oocytes aspirated per follicle (>13 mm) on the pre-trigger scan. The collection rates are then compared between clinicians working within the unit. Other indicators that could be recorded are the time taken for oocyte collection and the complication rate, although the incidence of bleeding and infection is so low that this is probably meaningless unless a large number of cases can be studied.

Additional guidance

The ESHRE working group on Ultrasound in ART [44] presents general recommendations for transvaginal oocyte pickup, and specific recommendations for its different stages, including before, during, and after the procedure. In addition, information is provided on equipment and materials, possible risks and complications, audit, and training. However, very few of

the recommendations are evidence-based and mostly consist of expert opinion of the members of the working group. It does provide a number of checklists, which would be helpful to a new unit starting up in ART, or as a structure for quality assurance.

A checklist prior to oocyte collection, like that used by pilots flying airplanes, has also been designed. It is encouraged that clinicians tick off each step to ensure that routine procedures are followed. A copy of this checklist is shown in [Table 50.2](#).

References

1. De Kretzer D, Dennis P, Hudson B, Leeton J, Lopata A, Outch K, Talbot J, Wood C. Transfer of a human zygote. *Lancet*. 1973;2:728–9.
2. Morgenstern LL, Soupart P. Oocyte recovery from the human ovary. *Fertil Steril*. 1972;23:751–8.
3. Berger MJ, Smith DM, Taymor ML, Thompson RS. Laparoscopic recovery of mature human oocytes. *Fertil Steril*. 1975;26:513–22.
4. Steptoe PC, Edwards RG. Laparoscopic recovery of preovulatory human oocytes after priming of ovaries with gonadotrophins. *Lancet*. 1970;1:683–6.
5. Renou P, Trounson AO, Wood C, Leeton JF. The collection of human oocytes for in vitro fertilization. I. An instrument for maximizing oocyte recovery. *Fertil Steril*. 1981;35:409–12.
6. Jones HW, Acosta AA, Garcia J. A technique for the aspiration of oocytes from human ovarian follicles. *Fertil Steril*. 1982;37:26–9.
7. Lenz S. Ultrasonic-guided follicle puncture under local anesthesia. *J In Vitro Fert Embryo Transf*. 1984;1:239–43.
8. Feichtinger W, Kemeter P. Laparoscopic or ultrasonically guided follicle aspiration for *in vitro* fertilization? *J In Vitro Fert Embryo Transf*. 1984;1244–9.
9. Kovacs GT, King C, Cameron I, Cushnahan L, Wood EC, Leeton JF, Renou P, Shekleton P, Levy S, Baker G. A comparison of vaginal ultrasonic-guided and laparoscopic retrieval of oocytes for *in vitro* fertilization. *Asia-Pacific J Obstet Gynaecol*. 1990;16:39–43.
10. Ditkoff E, Plumb J, Selick A, Saucer M. Anesthesia practice in the United States common to in vitro fertilization centers. *J Assisted Reprod Genet*. 1997;14:145–7.
11. Elkington N, Kehoe J, Acharya U. Recommendations for good practice for sedation in assisted conception. *Hum Fertil*. 2003;6: 77–80.
12. Vlahos NF, Giannakikou I, Vlachos A, Vitoratos N. Analgesia and anesthesia for assisted reproductive technologies. *Int J Gynaecol Obstet*. 2009;105:201–5.
13. Kwan I, Bhattacharya S, Knox F, McNeil A. Pain relief for women undergoing oocyte retrieval for assisted reproduction. *Cochrane Database Syst Rev*. 2013;1:CD004829.
14. Guasch E, Gómez R, Brogly N, Gilsanz F. Anesthesia and analgesia for transvaginal oocyte retrieval. Should we recommend or avoid any anesthetic drug or technique? *Curr Opin Anaesthesiol*. 2019;32(3):285–90.
15. Piroli A, Marci R, Marinangeli F, Paladini A, Di Emidio G, Giovanni Artini P, Caserta D, Tatone C. Comparison of different anaesthetic methodologies for sedation during in vitro fertilization procedures: Effects on patient physiology and oocyte competence. *Gynecol Endocrinol*. 2012;28:796–9.
16. van Os HC, Roozenburg BJ, Janssen-Caspers HA, Leerentveld RA, Scholtes MC, Zeilmaker GH. Alberda AT. Vaginal disinfection with povidon iodine and the outcome of *in-vitro* fertilization. *Hum Reprod*. 1992;7:349–50.
17. Hannoun A, Awwad J, Zreik T, Ghaziri G, Abu-Musa A. Effect of betadine vaginal preparation during oocyte aspiration in *in vitro* fertilization cycles on pregnancy outcome. *Gynecol Obstet Invest*. 2008;66:274–8.
18. Funabiki M, Taguchi S, Hayashi T, Tada Y, Kitaya K, Iwaki Y, Karita M, Nakamura Y. Vaginal preparation with povidone iodine disinfection and saline douching as a safe and effective method in prevention of oocyte pickup-associated pelvic inflammation without spoiling the reproductive outcome: Evidence from a large cohort study. *Clin Exp Obstet Gynecol*. 2014;41:689–90.
19. Younis JS, Ezra Y, Laufer N, Ohel G. Late manifestation of pelvic abscess following oocyte retrieval, for *in vitro* fertilization, in patients with severe endometriosis and ovarian endometriomata. *J Assist Reprod Genet*. 1997;14:343–6.
20. Horne R, Bishop CJ, Reeves G, Wood C, Kovacs GT. Aspiration of oocytes for *in-vitro* fertilization. *Hum Reprod Update*. 1996;2:77–85.
21. Scott RT, Hofmann GE, Muasher SJ, Acosta AA, Kreiner DK, Rosenwaks ZA. Prospective randomized comparison of single- and double-lumen needles for transvaginal follicular aspiration. *J In Vitro Fert Embryo Transf*. 1989;6:98–100.
22. Haydardedeoglu B, Cok T, Kilicdag EB, Parlakgumus AH, Simsek E, Bagis T. *In vitro* fertilization–intracytoplasmic sperm injection outcomes in single-versus double-lumen oocyte retrieval needles in normally responding patients: A randomized trial. *Fertil Steril*. 2011;95:812–4.
23. Steiner HP. Optimising techniques in follicular aspiration and flushing. In: *Technique of Minimal Stimulation in IVF—Milder, Mildest or Back to Nature*. Chivas-badiola A, Allahbadia GN (eds.). New Delhi, India: Jaypee Brothers Medical Publishers, pp. 98–102, 2011.
24. Kushnir VA, Kim A, Gleicher N, Barad DH. A pilot trial of large versus small diameter needles for oocyte retrieval. *Reprod Biol Endocrinol*. 2013;11:22–6.
25. Iduna Antigoni Buisman ET, de Bruin JP, Maria Braat DD, van der Steeg JW. Effect of needle diameter on pain during oocyte retrieval—a randomized controlled trial. *Fertil Steril*. 2021;115(3):683–91. <https://doi.org/10.1016/j.fertnstert.2020.06.040>.
26. Atzmon Y, Michaeli M, Aslih N, Ruzov O, Rotfarb N, Shoshan-Karchovsky E, Shalom-Paz E. Degenerative oocytes in the aspirated cohort are not due to the aspirating needle: A prospective randomized pilot study with sibling oocytes. *Reprod Sci*. 2021;28(7):1882–9. doi: [10.1007/s43032-020-00384-3](https://doi.org/10.1007/s43032-020-00384-3).
27. Hill MJ, Levens ED. Is there a benefit in follicular flushing in assisted reproductive technology? *Curr Opin Obstet Gynecol*. 2010;22:208–12.
28. Wongtra-Ngan S, Vutyavanich T, Brown J. Follicular flushing during oocyte retrieval in assisted reproductive techniques. *Cochrane Database Syst Rev*. 2010;8:CD004634.
29. Mendez Lozano DH, Brum Scheffer J, Frydman N, Fay S, Fanchin R, Frydman R. Optimal reproductive competence of oocytes retrieved through follicular flushing in minimal stimulation IVF. *Reprod Biomed Online*. 2008;16:119–23.
30. Georgiou EX, Melo P, Brown J, Granne IE. Follicular flushing during oocyte retrieval in assisted reproductive techniques. *Cochrane Database Syst Rev*. 2022 Nov 21;11(11):CD004634. doi: [10.1002/14651858.CD004634.pub4](https://doi.org/10.1002/14651858.CD004634.pub4).
31. Kohl Schwartz AS, Calzaferri I, Roumet M, Limacher A, Fink A, Wueest A, Weidlinger S, Mitter VR, Leeners B, Von Wolff M. Follicular flushing leads to higher oocyte yield in mono-follicular IVF: A randomized controlled trial. *Hum Reprod*. 2020;35(10):2253–61. <https://doi.org/10.1093/humrep/deaa165>
32. Calabre C, Schuller E, Goltzene MA, Rongières C, Celebi C, Meyer N, Teletin M, Pirrello O. Follicular flushing versus direct aspiration in poor responder IVF patients: A randomized prospective study. *Eur J Obstet Gynecol Reprod Biol*. 2020 May;248:118–122. doi: [10.1016/j.ejogrb.2020.03.003](https://doi.org/10.1016/j.ejogrb.2020.03.003).
33. Dahl SK, Cannon S, Aubuchon M, Williams DB, Robins JC, Thomas MA. Follicle curetting at the time of oocyte retrieval increases the oocyte yield. *J Assist Reprod Genet*. 2009;26:335–9.
34. Redding GP, Bronlund JE, Hart AL. The effects of IVF aspiration on the temperature, dissolved oxygen levels, and pH of follicular fluid. *J Assist Reprod Genet*. 2006;23:37–40.
35. Miller PB, Price T, Nichols JE Jr, Hill L. Acute ureteral obstruction following transvaginal oocyte retrieval for IVF. *Hum Reprod*. 2002;17:137–8.

36. Almog B, Rimon E, Yovel I, Bar-Am A, Amit A, Azem F. Vertebral osteomyelitis: A rare complication of transvaginal ultrasound-guided oocyte retrieval. *Fertil Steril.* 2000;73:1250–2.
37. De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko T, Scaravelli G, Smeenk J, Vidakovic S, Goossens V. European IVF-monitoring consortium (EIM) for the European society of human reproduction and embryology (ESHRE). ART in Europe, 2014: Results generated from European registries by ESHRE: The European IVF-monitoring consortium (EIM) for the European society of human reproduction and embryology (ESHRE). *Hum Reprod.* 2018;33(9):1586–1601.
38. Bennett SJ, Waterstone JJ, Cheng WC, Parsons J. Complications of transvaginal ultrasound-directed follicle aspiration: A review of 2670 consecutive procedures. *J Assist Reprod Genet.* 1993;10:72–7.
39. Nouri K, Walch K, Promberger R, Kurz C, Tempfer CB, Ott J. Severe haematoperitoneum caused by ovarian bleeding after transvaginal oocyte retrieval: A retrospective analysis and systematic literature review. *Reprod Biomed Online.* 2014;29:699–707.
40. Moini A, Malekzadeh F, Amirchaghmaghi E, Kashfi F, Akhoond MR, Saei M, Mirbolok MH. Risk factors associated with endometriosis among infertile Iranian women. *Arch Med Sci.* 2013;9:506–14.
41. Jayakrishnan K, Raman VK, Vijayalakshmi VK, Baheti S, Nambiar D. Massive hematuria with hemodynamic instability—Complication of oocyte retrieval. *Fertil Steril.* 2011;96:22–4.
42. Bentov Y, Levitas E, Silberstein T, Potashnik G. Cullen's sign following ultrasound-guided transvaginal oocyte retrieval. *Fertil Steril.* 2006;85:227.
43. Johnson NP, Mak W, Sowter MC. Surgical treatment for tubal disease in women due to undergo *in vitro* fertilisation. *Cochrane Database Syst Rev.* 2004;1:CD002125.
44. D'Angelo A, Panayotidis C, Amso N, Marci R, Matorras R, Onofriescu M, Turp AB, Vandekerckhove F, Veleva Z, Vermeulen N, Vlaisavljevic V, ESHRE Working Group on Ultrasound in ART. Recommendations for good practice in ultrasound: Oocyte pick up. *Hum Reprod Open.* 2019 Dec 10;2019(4):hoz025. doi: [10.1093/hropen/hoz025](https://doi.org/10.1093/hropen/hoz025).

LUTEAL PHASE SUPPORT IN ASSISTED REPRODUCTION TECHNIQUES

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Introduction

Luteal phase support (LPS) is one of the least personalized protocols within assisted reproduction techniques (ART). In general terms, exogenous progesterone (P4) is usually given for LPS after an embryo transfer (ET) [1], regardless the type of cycle followed, for proper endometrial preparation (modified natural, stimulated, or artificial cycle).

However, this standardized clinical practice for LPS does not take into account the endogenous P4 levels, which will be different regarding each type of cycle, nor the patient's absorption of exogenous P4, which will also depend on the dose and route of the P4 prescribed [2, 3].

On one hand, the hormonal profile triggered by each type of cycle is different and it may or may not lead to a luteal phase deficiency [2]. Luteal phase deficiency constitutes a situation in which the reached P4 levels are not enough for proper embryo implantation and maintenance of pregnancy [4]. In this event, LPS is always mandatory in order to ensure sufficient P4 levels.

On the other hand, different routes and doses of exogenous P4 administration will lead to different serum P4 behaviours [3]. Moreover, the exogenous P4 absorption capacity of each patient may be different, contributing to the unpredictability of this hormone serum profile.

Currently, the routine clinical practice in the majority of clinics is to follow the same LPS protocol in every ET, or at most to have one LPS protocol for each type of endometrial preparation cycle. This practice, however, is leaving behind patients' own characteristics, as well as the unknown luteal phase deficiency triggered in each specific case. Thus, a growing interest in an individualized LPS (iLPS) [5] has arisen over the past years, since each patient may need a particular route and dose of exogenous P4 depending on their features and the type of cycle they are following.

In fresh *in vitro* fertilization (IVF) cycles, which are the stimulated cycles, there is P4 production by the corpora lutea left behind after ovulation of several follicles. Despite this, minimum P4 levels may not be reached, leading to a luteal phase deficiency and the need for LPS [6]. Currently, there is no consensus on the best protocol for LPS in this type of cycle due to a lack of information on the triggered P4 hormonal profile. The endogenous P4 production may be different regarding the number of corpora lutea and the patient, leading to a very wide spectrum of serum P4 behaviour. However, a recent meta-analysis has proven that P4 supplementation for LPS, regardless its route of administration, increases success rates in IVF fresh cycles [7].

Over the past few years, serum P4 monitoring during the luteal phase of artificial cycles has been deeply studied in the literature. In this type of cycle, a single determination of serum P4 has been clearly related to pregnancy outcome, describing a minimum threshold level that must be reached in order to increase the chances of success [8, 9]. Moreover, it has been also demonstrated

that an iLPS in those patients that didn't reach the minimum P4 threshold was capable of increasing their success rates [10–12].

More research is needed in serum P4 monitoring in the luteal phase of fresh IVF cycles. In contrast to artificial cycles, the endogenous P4 source must be taken into account, as well as the ovarian response to stimulation and each patient's characteristics. There are some remaining questions which need to be answered:

- Is it necessary to LPS in fresh IVF cycles?
- Which is the optimal exogenous P4 dose in fresh IVF cycles?
- Is it worthy to measure serum P4 levels in the mid-luteal phase of fresh IVF cycles?
- Can LPS in fresh IVF cycles be individualized?

In the following sections, we will try to answer these questions, reviewing the literature available and giving an overview of the current status of LPS in fresh IVF cycles.

Luteal phase deficiency in fresh IVF cycles

The multi-follicular development induced by ovarian stimulation protocols results in a luteal phase deficiency in almost every IVF cycle. Gonadotropins given during the follicular phase stimulate the growth and development of a high number of oestradiol-producing follicles and, in consequence, the formation of a high number of progesterone-producing corpora lutea left behind after ovulation. Hence, supra-physiologic levels of these two steroids may inhibit LH secretion through a negative feedback mechanism at the hypothalamic–pituitary–gonadal axis [13, 14].

In addition to an impaired hormonal regulation, stimulation protocols may also affect the proper function of corpora lutea within the ovary. Corpora lutea have oestradiol and GnRH receptors, thus oestradiol and GnRH analogues binding to these structures favour luteolysis, impairing P4 production. Furthermore, corpus luteum function may be further compromised through the disruption and aspiration of granulosa cells during the oocyte pickup procedure [15].

Therefore, corpora luteal P4 production may be reduced during luteal phase in fresh IVF cycles, leading to luteal phase deficiency. Furthermore, this deficiency will be more or less pronounced regarding the type of trigger used for final oocyte maturation. The shorter half-life of the GnRH agonist, compared to hCG (human chorionic gonadotropin), lowers the risk of ovarian hyperstimulation syndrome (OHSS) at the expense of an impaired corpora luteal function and subsequent earlier luteolysis due to the hormonal axis suppression [16].

LPS in fresh IVF cycles can be performed using a wide variety of molecules and in different combinations. Exogenous P4, hCG, GnRH agonists, and oestradiol are among the available options. However, P4 is the most preferred exogenous hormone for LPS [1].

Progesterone sources in fresh IVF cycles

LPS has been extensively studied in artificial endometrial preparation cycles, in which the unique P4 source is of exogenous origin [17]. In contrast, fresh IVF cycles involve the presence of numerous progesterone-producing corpora lutea left behind after ovulation. The number of corpora lutea present in the ovary will correspond to the number of ovulated follicles, thus each luteinizing follicle will add P4 into circulation. Indeed, Arce et al. found a correlation between mono-follicular/multi-follicular response, as well as the number of follicles of medium and large size, and serum P4 levels measured six to nine days after hCG trigger in stimulated cycles without LPS [18].

In addition to this endogenous P4 production, and due to the proven luteal phase deficiency, LPS has been highly recommended in this type of cycle. Hence, patients' serum P4 levels will be of both endogenous and exogenous origin [2], thus the difficulty of monitoring this hormone level throughout luteal phase in fresh IVF cycles.

LPS in fresh IVF cycles is not standardized, and different protocols involving various routes and doses of exogenous P4 are used worldwide. In our case, our routine clinical practice is to give micronized vaginal P4 (MVP) (200 mg/12 hours) from the day after oocyte retrieval onwards, registering mean P4 levels of 93 ng/mL on the ET day (unpublished data). However, many other protocols involve other routes of P4 (oral, intramuscular, subcutaneous, etc.) and doses, making it impossible to generalize.

The majority of studies which will be deeply commented on in this chapter involve an ovarian stimulation protocol followed by an hCG triggering bolus. However, several authors have proposed innovative LPS protocols in IVF cycles following a GnRH-agonist triggering.

For instance, Kol and Segal, in 2020, proposed a progesterone-free LPS protocol, in which a bolus of hCG 48 hours after the oocyte pickup would be enough to support the luteal phase deficiency after triggering with the GnRH agonist. The election of 48 hours after the pickup is supported by the fact that it is the exact moment in which P4 levels begin to decline, thus rescuing the corpora lutea P4 production [19].

A similar protocol has been followed by Humaidan et al. in 2010 and 2013. These were not progesterone-free LPS protocols, as they also added vaginal P4 gel [20, 21]. In any case, the common aim of all these LPS protocols is to avoid an early luteal over-stimulation, favouring the P4 peak when needed, which is during the implantation window and not earlier, as it may happen with the hCG triggering.

Indeed, it is very important to properly define the duration of LPS in fresh IVF cycles, in order to optimize the exact moment of P4 peak and to maintain it until the luteal placental shift in P4 production. Regarding the start of LPS, Connell et al. suggested an optimal window between the day of oocyte retrieval and three days afterward for beginning LPS [22], while Mohammed et al. claimed that the optimal time to start P supplementation is the day after oocyte retrieval [7]. Regarding the end of LPS, some clinicians believe it could be discontinued on the day of the pregnancy test [23], while Petersen et al. proved that an extended LPS might have prevented miscarriage in those patients with later luteal placental shifts in P4 production [24]. Indeed, the majority of clinicians maintain it beyond the eighth week [25]. In our case, our routine clinical practice in fresh IVF cycles is to give MVP for LPS from the day after oocyte retrieval until the eighth week.

Progesterone behaviour during fresh IVF cycles

In the natural menstrual cycle, the endogenous LH peak upon ovulation induces follicular luteinization and the onset of P4 secretion by the resulting corpus luteum. Serum P4 levels, hence, continue to increase during the following days and peak around day 7 after ovulation, coinciding with the optimal window of implantation [26].

In the stimulated cycle, the hCG peak of exogenous origin that induces follicular luteinization drops sooner in time than the endogenous LH bolus of the natural cycle, probably due to the multi-follicular negative feedback previously mentioned [22] (Figure 51.1). Moreover, in GnRH-agonist-triggering cycles, this drop is even more pronounced [16]. Therefore, LPS in stimulated cycles is crucial for maintaining high P4 levels during the window of implantation, despite the premature hCG drop.

Regardless of the type of cycle, if implantation occurs and pregnancy is achieved, hCG produced by the implanted embryo rescues the corpora lutea, keeping P4 levels high until the luteal placental shift in P4 production [27].

But, what are the high P4 levels needed to succeed during the window of implantation? In IVF cycles, a minimum serum P4 threshold of 25.16–31.45 ng/mL during the mid-luteal phase has been proposed, as a reduced capability of the endometrium to sustain the early implantation has been evidenced with values below this point [28]. However, this contrast to the recently proven minimum serum P4 cut-off level on the ET day for artificial cycles when using MVP [9]; literature looking for this kind of prognostic threshold in fresh IVF cycles is more controversial. These studies involve different protocols for LPS and different points in the luteal phase established for hormonal determinations, which will be deeply reviewed in the following section.

Finally, serum P4 levels from the ET day onwards behave differently in fresh IVF and artificial cycles. In the latter, Labarta et al. have recently shown the evolution of serum P4 levels throughout luteal phase (measured on ET day, ET+4, ET+7, and ET+11) in artificial cycles using MVP for LPS. They have described different behaviours regarding the final pregnancy outcome, with ongoing pregnancies exerting a significant increasing trend in serum P4 levels over time [29]. In contrast, an analysis performed by Sonntag et al. in fresh IVF cycles using vaginal P4 for LPS described an initial serum P4 peak in the days following ET, which subsequently drops to finally increase gradually until ET+14 [30].

Therefore, whereas serum P4 levels in artificial cycles remain quite constant or with very slight changes [29], these hormonal levels in fresh IVF cycles suffer a clear drop around day 4 after ET to subsequently gradually increase as luteal phase progresses [30] (Figure 51.2). These results are partially concordant with the findings of Blakemore et al. in 2017, who have proven a clear difference in serum P4 levels between ET and ET+9 days in IVF cycles using intramuscular P4 for LPS [31].

These differences in serum P4 behaviour may be due to the endogenous P4 source present in fresh IVF cycles, which is absent in artificial cycles. The initial P4 peak around the ET day may be related to the coincidence in time of the endogenous P4 peak induced by the hCG bolus (Figure 51.1) and the exogenous source from LPS. Afterwards, endogenous P4 secretion gradually decreases, while the exogenous contribution continues, until hCG produced by the implanted embryo rescues corpora lutea and P4 levels begin to increase again.

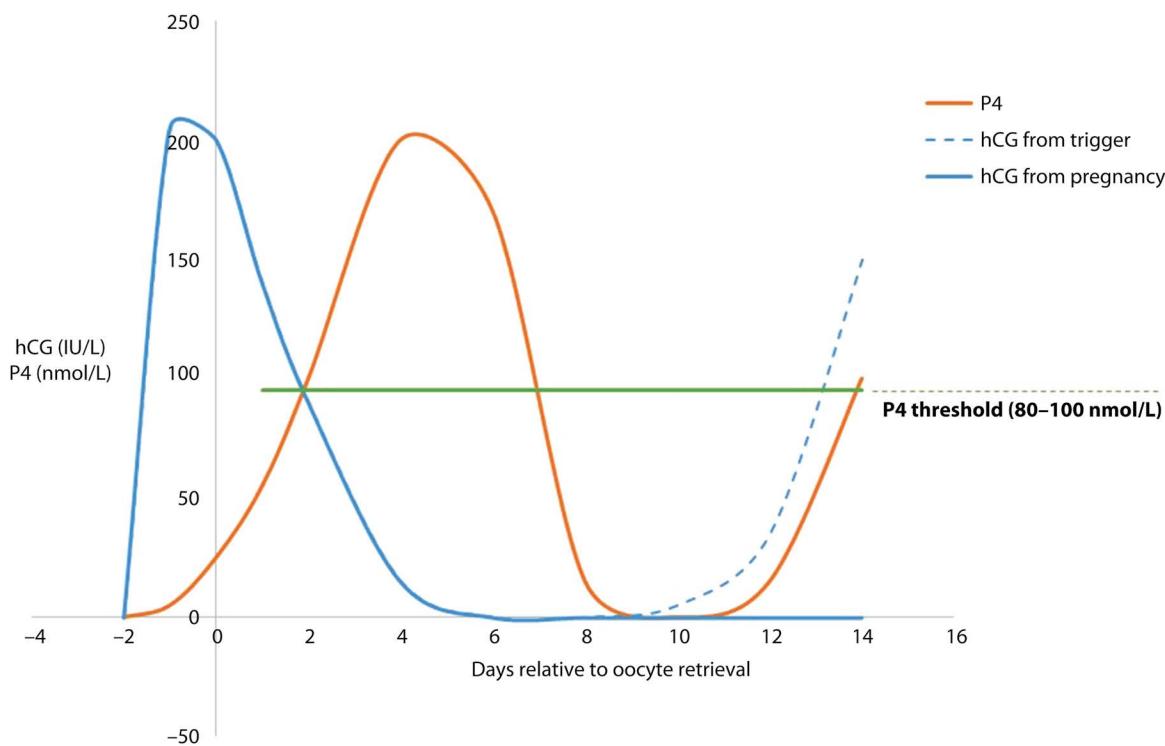


FIGURE 51.1 Representation of hCG and P4 levels from the day of hCG trigger until early pregnancy during an IVF stimulated cycle. Serum P4 levels are represented in nmol/L. (Adapted from [22].)

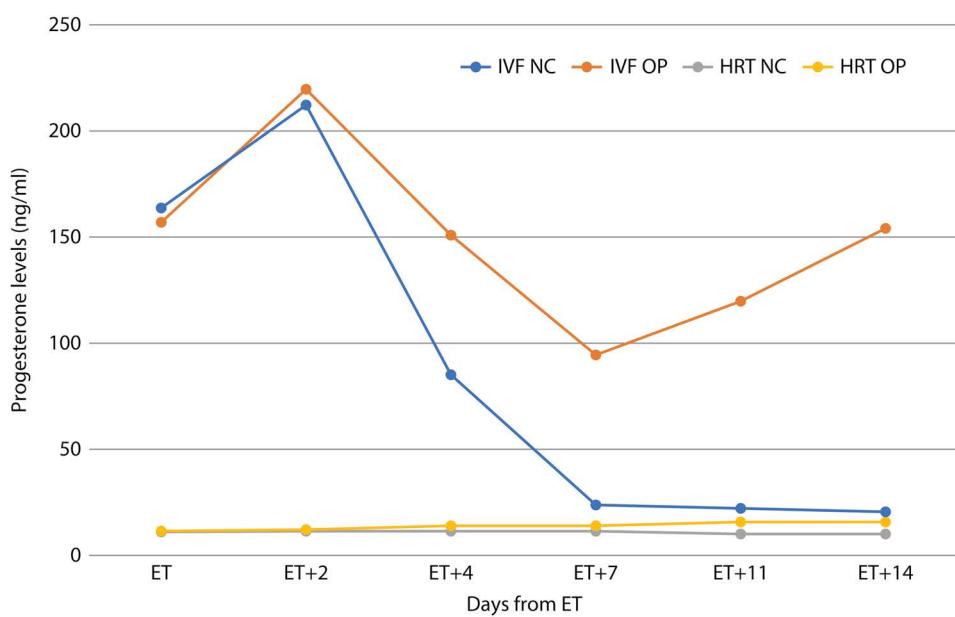


FIGURE 51.2 Progesterone behaviour from embryo transfer (ET) day onwards in IVF cycles versus HRT cycles, discriminating by non-conception (NC) and ongoing pregnancies (OP) and all of them using LPS. This figure was created considering data from Sonntag et al. for the IVF cycle [30] and Labarta et al. for the hormonal replacement treatment (HRT) cycle [29]. Progesterone levels on day ET+2 for the HRT cycle were estimated based on the levels registered on the previous and subsequent days, as they were not measured in this study.

It is interesting to point out the fact that the rise in serum P4 levels beyond ET+7 day supports the statement that this hormone is not only implicated in embryo implantation but also in pregnancy maintenance.

Luteal phase monitoring in fresh IVF cycles

Serum P4 levels monitoring in the mid-luteal phase of artificial cycles has allowed us to detect those “under-treated” patients and act in consequence, increasing their chances of success in the exact same cycle [10].

Taking this into account, it would be of special interest to also standardize luteal phase monitoring in fresh IVF cycles, in order to detect a minimum threshold to discriminate with a higher probability unsuccessful pregnancies. In this way, we may be able to translate these findings into an iLPS for this type of cycle. The proposed serum P4 threshold levels regarding success rates throughout the luteal phase in IVF cycles, which will be further reviewed in the following paragraphs, are represented in Figure 51.3.

The difficulty with fresh IVF cycles luteal phase monitoring is the presence of a variable and highly heterogeneous amount of progesterone-producing corpora lutea among patients. Indeed, a significant correlation has been found between follicle count at the end of stimulation and the mid-luteal progesterone concentration in a group of WHO-II women undergoing ovarian stimulation and ovulation induction with a bolus of hCG prior to intrauterine insemination, without LPS. In addition, serum P4 levels in the mid-luteal phase (measured six to nine days after hCG) were related to live birth rate (LBR), with increasing rates as we progress through the groups in which P4 was stratified (five groups from lower to higher hormonal levels) [18]. However, despite this proven association, they did not find any significant correlation between the number of large and medium-sized follicles and LBR [18], indicating that the number of corpora lutea was not the decisive parameter [28].

Hence, these findings suggest that the higher the mid-luteal P4 levels, the higher the success rates. However, interpatient and daytime variation in P4 levels might be considered. It has been demonstrated that daytime variation in P4 levels on day OPU+7 shows higher variability in women with high P4 levels ($>18.9 \text{ ng/mL}$), while it remains quite stable in those women with levels below this threshold [32]. Thus, P4 determinations below this threshold may be more reliable, making it easier to detect the group of women with lower prognosis.

However, this threshold of 18.9 ng/mL is too low to provide a reliable cut-off point in fresh IVF cycles. The formation of multiple corpora lutea and the use of a large bolus of hCG for final follicular maturation often raise these hormone levels over this point during the mid-luteal phase, either in the absence of LPS [18] or when using different protocols for LPS [20, 21, 33]. This fact has led to a proposed threshold of $25.2\text{--}31.4 \text{ ng/mL}$ during the mid-luteal phase, below which early pregnancy loss rates drop from about 80% to 10% [28].

In addition, unlike the study of Thomsen et al. [32], the majority of women undergoing an ET in the context of a stimulated cycle receive additional exogenous P4 for LPS, in order to prevent the already proven luteal phase deficiency. P4 levels monitoring in fresh IVF cycles with LPS has been studied in the context of different protocols for exogenous hormonal administration, adding extra difficulties to the establishment of a mid-luteal phase threshold point.

For instance, a retrospective study in fresh IVF cycles using intramuscular exogenous P4 for LPS found that both ongoing pregnancy rate (OPR) and LBR were positively related to P4 levels on ET and ET+9 days. Mean P4 levels were 46.9 and 34.2 ng/mL on days ET and ET+9, respectively. Hence, we see a trend to lower serum P4 levels as we progress into the late luteal phase. However, if this drop is pronounced, it may impair pregnancy outcome, while the ability to maintain relatively high serum P4 levels in the late luteal phase is a sign of good prognosis. Indeed, the most significant association with OPR and LBR was found for

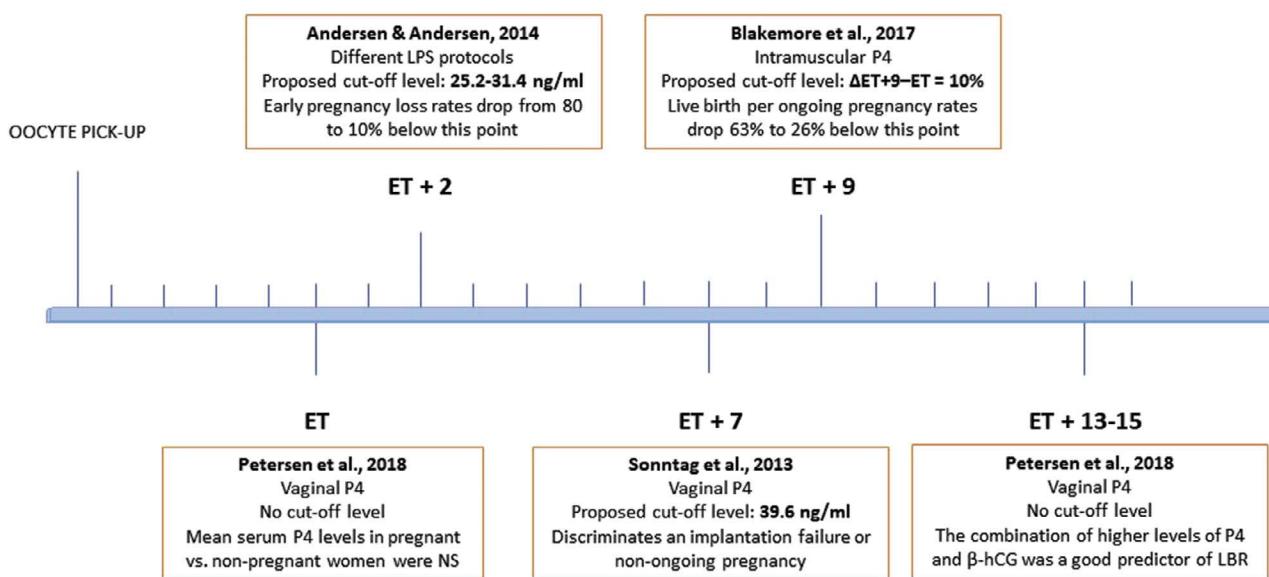


FIGURE 51.3 Representation of the different serum progesterone cut-off levels proposed by different studies regarding success rates throughout the luteal phase in IVF cycles. Abbreviations: NS, non-significant; ET, embryo transfer; P4, progesterone; LPS, luteal phase support; LBR, live birth rate; OPR, ongoing pregnancy rate.

the difference in serum P4 levels between ET+9 and ET days. The live birth per ongoing pregnancy rate dropped from 63% to 26% ($p < 0.001$) when this difference was greater than 10% [31].

In line with these results, serum P4 levels below 39.6 ng/mL on ET+7 day of stimulated cycles with vaginal P4 for LPS were able to detect an implantation failure or a non-going pregnancy in 92.3% of cases. In contrast, levels over this threshold predicted an ongoing pregnancy in 66.7% of cases, suggesting that a higher cut-off level might be calculated [30].

Contrary to these results, Petersen et al. found no correlation between serum P4 and oestradiol levels on ET day and the final cycle outcome in IVF cycles when using vaginal P4. These levels were only correlated to ovarian response. Despite this, the combination of high levels of P4 and β -hCG on ET+13–15 day were good predictors of LBR [24].

Increasing our knowledge about serum P4 levels behaviour throughout the luteal phase in stimulated cycles would help us with their monitoring, as well as to design an iLPS for each patient.

One interesting option to study endogenous P4 behaviour without giving up the LPS would be the administration of progestins such as dydrogesterone for LPS. This synthetic P4 does not cross-react with endogenous P4 when measured in the serum, allowing exclusive analysis of P4 of corpus luteum origin [34].

In any case, luteal monitoring of 17-OH P4 levels alone, a steroid produced exclusively by the corpus luteum, on OPU+7 of stimulated cycles when using vaginal P4 for LPS does not seem to offer a better insight into the corpus luteum function compared to the monitoring of total P4 levels [35].

Discussion

In the previous sections we have deeply described P4 sources, serum P4 behaviour, and the difficulties in serum P4 monitoring in fresh IVF cycles. Hence, we are now one step closer to trying to answer all the questions previously formulated:

- *Is LPS necessary in fresh IVF cycles?*

Yes, there is a luteal phase deficiency in almost every fresh IVF cycle.

- *What is the optimal exogenous P4 dose in fresh IVF cycles?*

There is no consensus, and it depends on the route of exogenous P4 administration.

- *Is it worthwhile to measure serum P4 levels in the mid-luteal phase of fresh IVF cycles?*

More studies are needed, but it seems that these hormone levels may be somehow related to pregnancy outcome.

- *Can LPS in fresh IVF cycles be individualized?*

Serum P4 behaviour in these types of cycles needs to be more deeply understood, along with how each patient's characteristics may be affecting these hormonal levels, in order to be able to design an iLPS.

In conclusion, LPS constitutes a mandatory protocol after an ET following a fresh IVF cycle. As many other protocols used in ART, LPS should be deeply studied in order to be able to individualize it to each patient's needs. To do so, luteal phase serum P4 levels should be assessed from a broader perspective, considering all the variables that may be affecting this hormonal profile behaviour. The understanding of this behaviour will help us in the search for the best LPS protocol for each patient in this type of cycle.

References

1. van der Linden M, Buckingham K, Farquhar C, Kremer JAM, Metwally M. Luteal phase support for assisted reproduction cycles. Cochrane Database Syst Rev. 2015;2015(7):10–3.
2. Labarta E, Rodríguez C. Progesterone use in assisted reproductive technology. Best Pract Res Clin Obstet Gynaecol. 2020;69:74–84.
3. Dashti S, Eftekhar M. Luteal-phase support in assisted reproductive technology: An ongoing challenge. Int J Reprod Biomed. 2021;19(9):761.
4. Practice Committee of the American Society for Reproductive Medicine. Current clinical irrelevance of luteal phase deficiency: A committee opinion. Fertil Steril. 2015;103(4):e27–32.
5. Labarta E. Relationship between serum progesterone (P) levels and pregnancy outcome: Lessons from artificial cycles when using vaginal natural micronized progesterone. J Assist Reprod Genet. 2020;37(8):2047–8.
6. Macklon NS, Fauser BC. Impact of ovarian hyperstimulation on the luteal phase. J Reprod Fertil Suppl. 2000;55:101–8.
7. Mohammed A, Woad KJ, Mann GE, Craigon J, Raine-Fenning N, Robinson RS. Evaluation of progestogen supplementation for luteal phase support in fresh in vitro fertilization cycles. Fertil Steril. 2019;112(3):491–502.e3.
8. Labarta E, Mariani G, Holtmann N, Celada P, Remohí J, Bosch E. Low serum progesterone on the day of embryo transfer is associated with a diminished ongoing pregnancy rate in oocyte donation cycles after artificial endometrial preparation: A prospective study. Hum Reprod. 2017;32(12):2437–42.
9. Labarta E, Mariani G, Paolelli S, Rodriguez-Varela C, Vidal C, Giles J, et al. Impact of low serum progesterone levels on the day of embryo transfer on pregnancy outcome: A prospective cohort study in artificial cycles with vaginal progesterone. Hum Reprod. 2021;36(3):683–92.
10. Labarta E, Mariani G, Rodríguez-Varela C, Bosch E. Individualized luteal phase support normalizes live birth rate in women with low progesterone levels on the day of embryo transfer in artificial endometrial preparation cycles. Fertil Steril. 2021;117(1):96–103.
11. Álvarez M, Gaggiotti-Marre S, Martínez F, Coll L, García S, González-Foruria I, et al. Individualised luteal phase support in artificially prepared frozen embryo transfer cycles based on serum progesterone levels: A prospective cohort study. Hum Reprod. 2021;36(6):1552–60.
12. Yarali H, Polat M, Mumusoglu S, Ozbek IY, Erden M, Bozdag G, et al. Subcutaneous luteal phase progesterone rescue rectifies ongoing pregnancy rates in hormone replacement therapy vitrified-warmed blastocyst transfer cycles. Reprod Biomed Online. 2021;43(1):45–51.
13. Smitz J, Devroey P, Van Steirteghem AC. Endocrinology in luteal phase and implantation. Br Med Bull. 1990;46(3):709–19.
14. Fauser BCJM, Devroey P. Reproductive biology and IVF: Ovarian stimulation and luteal phase consequences. Trends Endocrinol Metab. 2003;14(5):236–42.
15. Tavaniotou A, Smitz J, Bourgain C, Devroey P. Ovulation induction disrupts luteal phase function. Ann N Y Acad Sci. 2001;943:55–63.
16. Humaidan P, Engmann L, Benadiva C. Luteal phase supplementation after gonadotropin-releasing hormone agonist trigger in fresh embryo transfer: The American versus European approaches. Fertil Steril. 2015;103(4):879–85.
17. Groenewoud ER, Cohlen BJ, Macklon NS. Programming the endometrium for deferred transfer of cryopreserved embryos: Hormone replacement versus modified natural cycles. Fertil Steril. 2018;109(5):768–74.
18. Arce JC, Balen A, Plattein P, Pettersson G, Andersen AN. Mid-luteal progesterone concentrations are associated with live birth rates during ovulation induction. Reprod Biomed Online. 2011;22(5):449–56.

19. Kol S, Segal L. GnRH agonist triggering followed by 1500 IU of hCG 48 h after oocyte retrieval for luteal phase support. *Reprod Biomed Online.* 2020;41(5):854–8.
20. Humaidan P, Ejdrup Bredkjær H, Westergaard LG, Yding Andersen C. 1,500 IU human chorionic gonadotropin administered at oocyte retrieval rescues the luteal phase when gonadotropin-releasing hormone agonist is used for ovulation induction: A prospective, randomized, controlled study. *Fertil Steril.* 2010;93(3):847–54.
21. Humaidan P, Polyzos NP, Alsbjerg B, Erb K, Mikkelsen AL, Elbaek HO, et al. GnRHa trigger and individualized luteal phase hCG support according to ovarian response to stimulation: Two prospective randomized controlled multi-centre studies in IVF patients. *Hum Reprod.* 2013;28(9):2511–21.
22. Connell MT, Szatkowski JM, Terry N, DeCherney AH, Propst AM, Hill MJ. Timing luteal support in assisted reproductive technology: A systematic review. *Fertil Steril.* 2015;103(4):939–946.e3.
23. Pan SP, Chao KH, Huang CC, Wu MY, Chen MJ, Chang CH, et al. Early stop of progesterone supplementation after confirmation of pregnancy in IVF/ICSI fresh embryo transfer cycles of poor responders does not affect pregnancy outcome. *PLoS One.* 2018;13(8):1–11.
24. Petersen J, Andersen A, Klein B, Helmggaard L, Arce J. Luteal phase progesterone and oestradiol after ovarian stimulation : Relation to response and prediction of pregnancy. *Reprod Biomed Online.* 2018;36:427–34.
25. Vaisbuch E, de Ziegler D, Leong M, Weissman A, Shoham Z. Luteal-phase support in assisted reproduction treatment: Real-life practices reported worldwide by an updated website-based survey. *Reprod Biomed Online.* 2014 Mar 1;28(3):330–5.
26. Devoto L, Fuentes A, Kohen P, Céspedes P, Palomino A, Pommer R, et al. The human corpus luteum: Life cycle and function in natural cycles. *Fertil Steril.* 2009;92(3):1067–79.
27. Lawrenz B, Coughlan C, Fatemi HM. Individualized luteal phase support. *Curr Opin Obstet Gynecol.* 2019;31(3):177–82.
28. Yding Andersen C, Vilbour Andersen K. Improving the luteal phase after ovarian stimulation: Reviewing new options. *Reprod Biomed Online.* 2014;28(5):552–9.
29. Labarta E, Rodríguez-Varela C, Mariani G, Bosch E. Serum progesterone profile across the mid and late luteal phase in artificial cycles is associated with pregnancy outcome. *Front Endocrinol (Lausanne).* 2021;12.
30. Sonntag B, Loebbecke KC, Nofer JR, Kiesel L, Greb RR. Serum estradiol and progesterone in the mid-luteal phase predict clinical pregnancy outcome in IVF/ICSI cycles. *Gynecol Endocrinol.* 2013;29(7):700–3.
31. Blakemore JK, Kofinas JD, McCulloh DH, Grifo J. Serum progesterone trend after day of transfer predicts live birth in fresh IVF cycles. *J Assist Reprod Genet.* 2017;34(3):339–43.
32. Thomsen LH, Kesmodel US, Andersen CY, Humaidan P. Daytime variation in serum progesterone during the mid-luteal phase in women undergoing in vitro fertilization treatment. *Front Endocrinol (Lausanne).* 2018;9.
33. Humaidan P, Bredkjær HE, Bungum L, Bungum M, Grøndahl ML, Westergaard L, et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomized study. *Hum Reprod.* 2005;20(5):1213–20.
34. Neumann K, Depenbusch M, Schultze-Mosgau A, Griesinger G. Characterization of early pregnancy placental progesterone production by use of dydrogesterone in programmed frozen-thawed embryo transfer cycles. *Reprod Biomed Online.* 2020 May 1;40(5):743–51.
35. Thomsen LH, Humaidan P, Erb K, Overgaard M, Andersen CY, Kesmodel US. Mid-luteal 17-OH progesterone levels in 614 women undergoing IVF-treatment and fresh embryo transfer—Daytime variation and impact on live birth rates. *Front Endocrinol (Lausanne).* 2018;9:690.

TREATMENT STRATEGIES IN ASSISTED REPRODUCTION FOR THE POOR-RESPONDER PATIENT

Ariel Weissman and Colin M. Howles

Overview

In a spontaneous menstrual cycle, only one follicle out of a cohort of 10–20 usually completes maturation and ovulates to release a mature oocyte. The aim of controlled ovarian stimulation (COS) in assisted reproductive technology (ART) protocols is to overcome the selection of a dominant follicle and to allow the growth of a cohort of follicles. This strategy leads to an increase in the number of oocytes and hence embryos available for transfer and cryopreservation, thereby increasing the chance of transferring viable embryos. However, the chance of pregnancy and also live birth begins to dramatically decline after the age of 35 years, and successful treatment for these patients continues to be a major challenge in ART programs.

Since the last edition of this book, there has been a further acceleration of research, trials, and data on this patient population that becomes increasingly important as the average treatment age of women coming into ART continues to increase. In view of this, we have allocated in this edition separate new chapters to cover in detail subjects such as the POSEIDON stratification, protocols such as mild stimulation, DuoStim, adjuvants for poor responders (testosterone, DHEA, growth hormone, CoQ10), as well as innovative therapies including *in vitro* activation (IVA) and stem cells, etc.

In this chapter, we will historically review and update strategies aimed at augmenting follicular recruitment, oocyte yield, and ultimate desired clinical outcomes following *in vitro* fertilization (IVF) treatment among women identified as poor responders.

Introduction

The human ovary has a finite number of non-growing follicles (NGFs) established before birth that decline with increasing age, culminating in menopause at age 50–51 years. For 95% of women, only 12% of their pre-birth NGF population is present by the age of 30 years, declining to only 3% by the age of 40 years [1]. This provides the basis for decline in female fecundity with increasing age. This decline in fecundity can be based on a variety of age-related conditions, including an increase in gynaecological disorders such as endometriosis or fibroids, an increase in ovulatory disorders due to effects on the hypothalamic–pituitary–ovarian axis, or a compromised uterine vascular supply that may impede implantation [2]. Spontaneous conception is rare in women >45 years of age: a study carried out in orthodox Jewish sects that are proscribed from using contraceptives showed that natural pregnancies and deliveries after the age of 45 years constitute only 0.2% of total deliveries, and >80% of these are in grand multiparas [3]. Similar findings have been described in Bedouin women as well [4]. In infertile couples, IVF may be a reasonable option for such women of advanced maternal age (AMA) who are aged >40 years, but at the age of ≥45 years, deliveries are a rare event [5].

The peak number of oocytes present in the human ovary occurs during fetal gestation, and follicles are continually lost thereafter through the mechanism of apoptosis, a process known as atresia [6]. A cohort of growing follicles is recruited each month, and the cohort enters the final stages of follicle maturation during the first half of the menstrual cycle. This maturation phase is gonadotropin dependent. Painstaking histological and *in vitro* studies carried out by Gougeon suggest that follicles require a period of approximately 70 days from the time they enter the preantral stage (0.15 mm) to reach a size of 2 mm [7]. These 2-mm follicles have very low steroidogenic activity, and they are impervious to cyclic follicle-stimulating hormone (FSH) and luteinizing hormone (LH) changes in terms of granulosa cell (GC) proliferation. Over a four- to five-day period during the late luteal phase, follicles that are 2–5 mm in diameter enter a recruitment stage, and cyclic changes in FSH drive the development of the follicle and the proliferation of GCs; GC aromatase activity is not affected during this stage. Thus, as the follicle develops, it becomes increasingly responsive to gonadotropins.

From the perspective of treatment management, this means that in order to influence the size of the recruitable pool of follicles, it would be necessary to “boost” continued healthy follicle development over a protracted period of time (≥70 days). However, gonadotropins play a role only during the phases of recruitment and final follicular maturation, which occur over the last 20 days or so of this 70-day period. Therefore, extrapolating from knowledge about basic physiology, different agents would be required at different times in order to successfully overcome the age-related decline in follicle numbers.

Women who postpone childbearing until their late 30s or early 40s are therefore frequently faced with the distressing realization that their chance of achieving a pregnancy is significantly reduced, and that they may require the help of ART, with further complex difficulties that can jeopardize their quest for successful conception. In Europe, for the year 2015, women undergoing IVF or intracytoplasmic sperm injection (ICSI) procedures in the age group >39 years represented approximately 18.4% and 20.3%, respectively, of those undergoing aspirations [8]. A number of different variables can affect success rates in ART, and the negative impact of increasing age is one feature that is well recognized. Not only does the response to stimulation steadily deteriorate, requiring larger amounts of gonadotropins, but also the cancellation rate is higher, and there is a significant increase in the rate of miscarriage.

Data from the United States (Centers for Disease Control [CDC] 2019 report on ART success rates) [9] clearly show that the potential for successful delivery of a live birth/embryo transfer from autologous oocytes starts to decrease rapidly in women >35 years, whereas, in donor cycles, the live birth delivery rate per embryo transfer stays above 40%, irrespective of the age of the recipient (Figure 52.1). Previously, the CDC 2013 report also

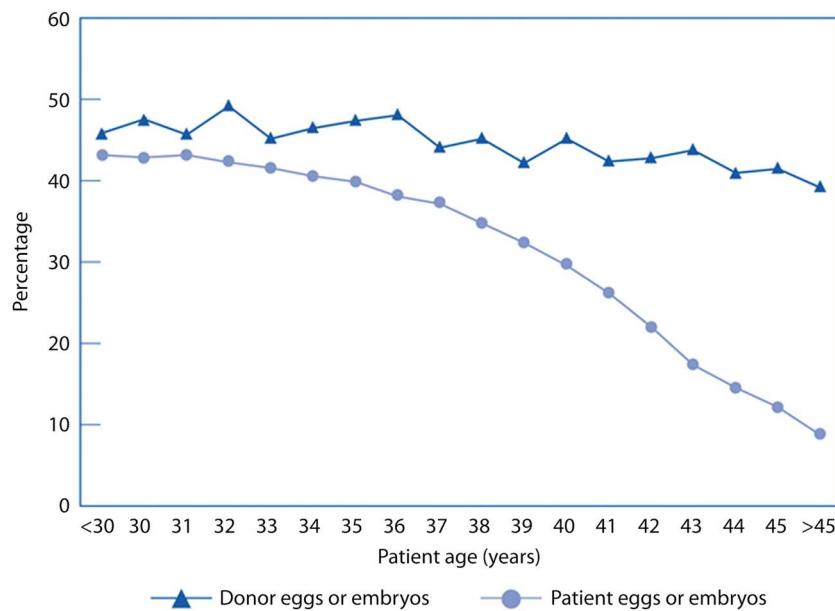


FIGURE 52.1 Percentage of embryo transfers that resulted in live birth delivery, by patient age and egg or embryo. (United States, Centers for Disease Control and Prevention data, 2019 [9].)

documented the increased incidence of pregnancy loss that is related to increased maternal age, going from less than 15% in women ≤ 36 years of age, increasing rapidly among women in their late 30s to reach 29% at 40 years of age, and over 50% in women ≥ 44 years. These data suggest that the lower age limit to defining women of AMA should be considered as ≥ 35 years [10].

Although chronological age is the most important predictor of ovarian response to stimulation, the rate of reproductive aging and ovarian sensitivity to gonadotropins varies considerably among individuals [11]. Biological and chronological age are not always equivalent, and biological age is more important in predicting the outcome of ART [11]. Biological aging often renders the ovaries increasingly resistant to gonadotropin stimulation, with the result that the number of oocytes harvested may be very low.

Any strategy that might enhance the efficacy of treatment for these women would be of great benefit, and different areas of research have recently been explored, such as the use of pharmacogenomics to assess response to gonadotropin stimulation, manipulating the endocrinology of the treatment cycle, and screening of embryos for aneuploidy.

Definitions and terminology

Over the years, a plethora of papers on different aspects of the pathogenesis and management of poor ovarian response (POR) have been published. One of the major problems in comparing these studies was the lack of a uniform definition of a poor response. The considerable heterogeneities in the definition of POR (inclusion criteria, outcome measures, etc.) made it almost impossible to develop or assess any protocol to improve the outcome [12, 13]. To this effect, the European Society of Human Reproduction and Embryology (ESHRE) working group attempted to standardize the definition of POR to stimulation in a simple and reproducible manner (the Bologna consensus) [14]. POR to ovarian stimulation usually indicates a reduction in follicular response, resulting in a reduced number of retrieved oocytes. The consensus definition recommends that two of the following three features should be

present for a diagnosis of POR: (1) AMA (≥ 40 years) or other risk factor for POR; (2) a previous POR (≤ 3 oocytes with a conventional stimulation protocol); and (3) an abnormal ovarian reserve test (ORT) (i.e. antral follicle count [AFC] $<5-7$ follicles or anti-Müllerian hormone [AMH] $<0.5-1.1$ ng/mL [3.57-7.85 pmol/L]). Two episodes of POR after maximal stimulation were considered sufficient to define a patient as a poor responder in the absence of AMA or abnormal ORT. Patients of AMA with an abnormal ORT may be more properly defined as “expected poor responders.” Although subject to initial criticism about its validity in defining a homogenous population [15], subsequent studies have established its validity that the various subgroups of the Bologna criteria poor responders have a uniform poor prognosis [16, 17]. More recently, a new stratification system (POSEIDON grouping [18, 19]; <https://www.groupbyseidon.com>) has been put forward for classifying infertility patients with confirmed or expected low ovarian response to exogenous gonadotropins. Specifically, four subgroup categories have been created based on quantitative and qualitative parameters, namely, (i) the age of the patient and the expected aneuploidy rate; (ii) ovarian biomarkers (i.e. AFC and AMH), and (iii) the ovarian response of the patient provided a previous cycle of stimulation had been carried out. In the latter, a “suboptimal response” was defined as the retrieval of four to nine oocytes despite adequate pre-stimulation ovarian parameters, as it is associated at any given age with a significantly lower live birth rate compared with normal responders, i.e. those with 10–15 oocytes. And a “poor response” was defined as the retrieval of fewer than four oocytes despite adequate pre-stimulation ovarian parameters. For more detail see chapter 53.

Assessment and prediction of ovarian response to stimulation

The ability to accurately assess and predict ovarian response would reduce the burdens imposed by failure because of inadequate response to stimulation. Unfortunately, the response to stimulation cannot be reliably predicted, even for young patients

with no evidence of endocrine disorders. Parameters that have been identified as exerting an influence include age [20, 21], cause of infertility [11], body weight [21], and body mass index (BMI) [21]. Ovarian characteristics have also been assessed by ultrasound, such as the number and size of antral follicles, ovarian volume, and ovarian vascular resistance measured by Doppler ultrasound.

There is a clear correlation between the number of antral follicles (defined as ≥ 2 mm to ≥ 10 mm) seen at the beginning of the follicular phase during a natural cycle (NC) and subsequent ovarian response to stimulation. However, there is as yet no consensus of agreement regarding the minimum number of antral follicles below which an influence can be seen [22–28]; a minimum of fewer than five follicles of 2–5 mm in diameter has been suggested as a predictive parameter [27]. One of the major reasons for this was a lack of standardized definition for assessment of the AFC, whose accuracy of measurement is highly operator dependent [26]. Klinkert et al. [27] suggest that patients with an AFC of fewer than five follicles of 2–5 mm in diameter are expected to have a poor response, and in a randomized controlled trial (RCT), they demonstrated that doubling the starting doses of gonadotropins does not lead to an improvement in response for these patients during IVF treatment [28]. In this study of 52 patients, more than half were aged >40 years, and 13 had basal FSH levels >15 IU/L.

Basal hormone assessment at the start of the follicular phase has been used to predict ovarian response, including FSH [29–35], oestradiol (E2) [34, 35], and inhibin-B [35–40]. AMH is an accurate marker of ovarian reserve and oocyte yield [41–45]. Circulating levels of AMH decline with increasing biological ovarian age but remain relatively stable throughout each menstrual cycle [45, 46], leading to it being measurable with accuracy at any time during the cycle. A comparison of AMH and FSH as predictors of retrieved oocyte numbers showed that AMH was clearly superior at predicting ovarian response [47]. Moreover, a meta-analysis comparing AMH and AFC showed that AMH had the same level of accuracy and clinical value as AFC for the prediction of poor and hyper-response in IVF [48, 49]. A prospective cohort study of 538 patients undergoing their first ART cycle with differential COS strategies based on an AMH measurement showed that AMH was associated with oocyte yield and that a

low AMH (1 to <5 pmol/L) was associated with a reduced clinical pregnancy rate [50]. Similarly, other investigators showed that AMH-based prediction of ovarian response was independent of age and polycystic ovary syndrome (PCOS) in 165 patients undergoing a first COS cycle for ART [51]. AMH was a significantly better predictor of poor response compared with FSH but not AFC. Various AMH cut-off values to predict a poor response have been explored. It has been suggested that an AMH cut-off level of <1.0 ng/mL (7.14 pmol/L) may have modest sensitivity and specificity in predicting a poor response to COS [51]. For further details, see Figure 52.2.

Individualized, AMH-guided treatment protocols were shown to significantly improve IVF outcomes whilst reducing adverse effects and costs compared with conventional treatment in a retrospective study of 796 women [52]. The incidence of ovarian hyperstimulation syndrome (OHSS) was also significantly lower with AMH-tailored versus conventional treatment. An age-related AMH nomogram is available for pre-treatment patient counselling [45].

There have been attempts to develop models for ovarian response based upon algorithms made up of multiple predictive factors. For instance, Popovic-Todorovic and colleagues developed a scoring system for calculating the FSH starting dose, based on four predictors: the total number of antral follicles, total Doppler score, serum testosterone (T) levels, and smoking habit [24]. This model was tested prospectively in a two-site clinical study, in which an ongoing pregnancy rate of 36.6% was reported using the algorithm to assign starting FSH doses of between 100 and 250 IU, compared with an ongoing pregnancy rate of 24.4% with a standard protocol using 150 IU FSH [53]. Another algorithm to predict the recombinant human FSH (r-FSH; follitropin- α) starting dose has been described but is only applicable to young (<35 years of age), normogonadotropic women [54]. The four factors identified as significantly predictive of ovarian response were baseline serum FSH levels, BMI, age, and AFC. Prognostic testing for ovarian reserve is described in further detail in Chapter 38.

Given that AFC and AMH have the best predictive accuracy among other ovarian reserve markers, current therapeutic strategies have been proposed using either of these tests to choose the ideal protocol. Such tailored treatment protocols maximize IVF

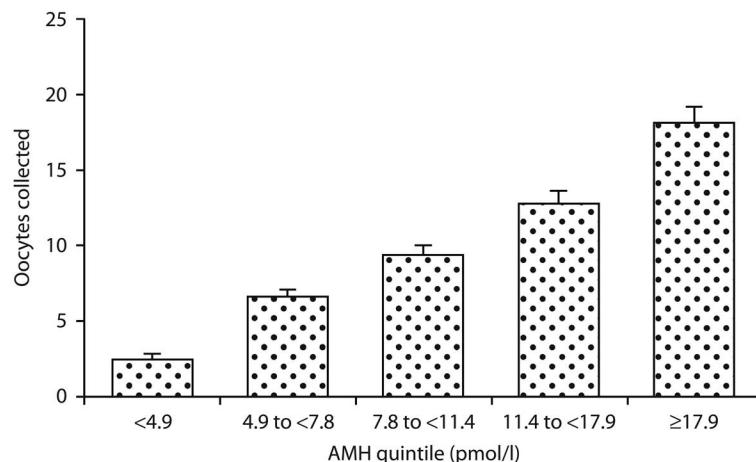


FIGURE 52.2 Mean oocyte yield per AMH quintile. Values are mean \pm standard error of the mean (SEM) [41]. Abbreviation: AMH, anti-Müllerian hormone.

outcomes whilst reducing avoidable risks such as cycle cancellation and OHSS [55].

This chapter will focus on a review of classic and specialized protocols designed for poor-responder patients, as well as on hormonal and pharmacologic manipulations that are expected to improve ovarian response. A large variety of strategies have been developed to improve outcome in patients with diminished ovarian reserve. There is no established intervention or treatment protocol for poor responders [56, 57]. Indeed, all of the currently available COS protocols have been used, with or without modifications, for the treatment of poor responders. Unfortunately, each of these approaches has achieved only limited success [56–61].

High-dose gonadotropins

It is generally believed that the dose of gonadotropins should be adjusted upwards in an attempt to overcome the age-related decline in ovarian response to FSH stimulation. Patients who responded poorly to conventional doses (150–225 IU of FSH) may produce more follicles when given 300–450 IU or even 600 IU per day. It is expected that an enhanced response would lead to an increased number of oocytes retrieved, number of available embryos, and, ultimately, higher pregnancy and live birth rates [62, 63]. These expectations, however, are not always met, and this strategy is often of limited effectiveness.

Although higher circulating levels may be achieved by increasing the quantity of gonadotropins being administered, at some point saturation kinetics are attained [62, 64] and the ovarian response is determined more by the number of follicles available for recruitment than by circulating gonadotropin levels. This is of particular importance, since poor responders generally have markedly diminished numbers of follicles available for recruitment, as reflected in their low AFC.

Very few studies have been conducted on the effects of increasing the dose of gonadotropins in poor responders. There are currently two published RCTs evaluating the efficacy of high gonadotropin starting doses in presumed poor responders [65, 66]. The first RCT randomized women undergoing IVF with an AFC <12 to receiving daily fixed doses of 300 IU versus 375 IU versus 450 IU of recombinant FSH using a microdose flare protocol [65]. There was no significant difference in the number of mature oocytes retrieved, cycle cancellation, number of embryos transferred, and clinical pregnancy rates between the three arms. In the more recent RCT involving 356 women categorized as being at risk of POR based on age <41 years with basal FSH >10 IU/L, AMH <1 ng/mL, AFC ≤8, or a previous IVF cycle with ≥300 IU/day gonadotropin that resulted in a cycle cancellation, fewer than eight follicles, or fewer than five oocytes, these women were randomized to 450 IU versus 600 IU of gonadotropin daily in a microdose agonist flare-up protocol [66]. The study showed no significant differences in the number of mature oocytes, fertilization rate, implantation rate, and clinical pregnancy rate between the two groups. The commencement of both of these studies predated the ESHRE consensus, and hence POR was not defined according to the Bologna criteria/POSEIDON Grouping. However, evidence from these studies suggests that very high gonadotropin doses of >300 IU daily are unlikely to be beneficial.

An interesting question is whether it is possible to rescue a cycle with initial poor response by doubling the gonadotropin dose after stimulation has already started. An RCT [67] evaluated the effect of doubling the human menopausal gonadotropin

(hMG) dose in the current cycle in which the ovarian response after five days of ovarian stimulation with 225 IU/day was considered “low.” No effect of doubling the hMG dose was noted with regard to the length of ovarian stimulation, peak E2 values, number of follicles >11, and >14 mm in diameter on the day of human chorionic gonadotropin (hCG) administration, number of cancelled cycles, number of oocytes retrieved, and the number of patients with three or fewer oocytes retrieved. It was concluded that doubling the hMG dose in the course of an IVF cycle is not effective at enhancing ovarian response. This is in accordance with current understanding of follicular growth dynamics, which states that follicular recruitment occurs only in the late luteal phase of the previous and early follicular phase of the current menstrual cycle.

In summary, increasing the starting dose of gonadotropins in poor responders is a rational approach that is widely practiced. A common starting dose would be at least 300 IU/day. Nevertheless, further dose increments are of limited effectiveness, and clinically meaningful improvements are only rarely obtained with doses >300 IU/day. The ESHRE Guideline Group on Ovarian Stimulation has recently published similar recommendations [68].

Gonadotropin-releasing hormone agonists in the treatment of poor responders

Long gonadotropin-releasing hormone agonist protocols

The use of gonadotropin-releasing hormone agonists (GnRH α) has gained widespread popularity and most ART programs frequently use this approach for COS. A meta-analysis of RCTs and quasi-RCTs showed that use of GnRH α reduced cancellation rates, increased the number of oocytes retrieved, and improved clinical pregnancy rates per cycle commenced and per embryo transfer (ET), compared with conventional stimulation regimens without the use of GnRH analogues [69]. The aim of the long protocol is to achieve pituitary downregulation with suppression of endogenous gonadotropin secretion before stimulation with exogenous gonadotropins. Once pituitary downregulation and ovarian suppression are achieved, ovarian stimulation with exogenous gonadotropins is commenced, while GnRH α administration is continued concomitantly until the day of hCG administration. In the general IVF population, the long protocol has been found to be superior in terms of efficacy compared with the short protocol [70] and was therefore used most frequently for many years, until the introduction of GnRH antagonist into clinical practice. However, the matter of which GnRH α protocol is preferable in poor responders remains controversial.

Downregulation of the hypothalamic–pituitary–ovarian axis prior to gonadotropin therapy is often associated with prolongation of the follicular phase and a significant increase in the dosage of gonadotropins required to achieve adequate follicular development. The extent of this increase is far greater than what could be attributed to simply delaying hCG administration to the point where a larger cohort of homogeneously well-synchronized large follicles are present. Moreover, in some relatively young patients with normal ovarian reserve, it was difficult to induce any ovarian response in the presence of pituitary downregulation, even with very large doses of exogenous gonadotropins [71–75]. Normal ovarian function was restored in these patients after withdrawal of the GnRH α , with subsequent normal response to hMG [73, 74]. These early observations indicated that GnRH α may induce

a state of ovarian hypo-responsiveness and raised doubt on the efficacy of the long protocol for poor responders.

Several theories have been suggested in an attempt to explain the dramatic (often twofold) increase in exogenous gonadotropin requirements during pituitary downregulation:

1. Diminished circulating endogenous gonadotropin levels [71, 72]
2. Altered biologic activity of endogenous gonadotropins [76–78]
3. Interference with follicular recruitment [79]
4. Direct ovarian inhibition effects by GnRHa [80, 81]

It has been well established that there is a dose-dependent duration of ovarian suppression after single implant injections of GnRHa, and that in a suppressed pituitary gland the dose needed to maintain suppression gradually decreases with the length of treatment [82]. This supports the concept of step-down GnRHa protocols, where the dose of the agonist is decreased once the criteria for ovarian suppression have been achieved. Furthermore, the minimal effective dose for sufficient pituitary suppression with GnRHa has not been thoroughly studied before their actual introduction to clinical practice. Regarding triptorelin, for example, Janssens et al. [83], in a prospective, placebo-controlled, double-blind study, demonstrated that daily administration of 15 µg of triptorelin is sufficient to prevent a premature LH surge, and that 50 µg is equivalent to 100 µg in terms of IVF results.

In an attempt to maximize ovarian response without losing the benefits of GnRHa downregulation, Feldberg et al. [84] introduced the use of the mini-dose GnRHa protocol in poor responders. They found that patients with elevated basal FSH levels who received daily triptorelin 100 µg subcutaneously (SC) from the mid-luteal phase until menstruation, and 50 µg thereafter, had higher peak E2 levels, more oocytes recovered, and more embryos transferred. They also noted a trend toward improved pregnancy and implantation rates and a lower spontaneous abortion rate.

Olivennes et al. [85] studied 98 IVF patients with a high basal FSH concentration who were previously treated by the long protocol with a GnRHa in a depot formulation. The same patients received SC leuprolide acetate (LA) 0.1 mg/day from cycle day 21, reducing it to 0.05 mg/day upon downregulation. The comparison was made using the previous IVF cycle of the same patient as a control. The use of a low-dose agonist protocol resulted in significantly reduced gonadotropin requirements, a shorter duration of stimulation, a higher E2 concentration on stimulation day 8, a higher number of mature oocytes, and a higher number of good-quality embryos. The cancellation rate was lower (11% vs 24%). Kowalik et al. [80] have demonstrated that lowering the dose of LA resulted in a faster E2 rise and higher mean peak E2 level. The higher E2 levels were obtained with a lower total gonadotropin dose. The oocyte yield was not affected. It was concluded that lowering the dosage of LA allows higher E2 response, which suggests an inhibitory *in vivo* effect of LA on ovarian steroidogenesis. Davis and Rosenwaks [75] reported similar results using a low-dose LA protocol.

Weissman et al. [86] prospectively compared two stimulation protocols specifically designed for poor responder patients. Sixty poor responders who were recruited on the basis of response in previous cycles received either a modified flare-up protocol in which a high dose of triptorelin (500 µg) was administered for the first four days followed by a standard dose (100 µg), or

a mini-dose long protocol in which 100 µg triptorelin was used until pituitary downregulation, after which the triptorelin dose was halved during stimulation. Twenty-nine cycles were performed with the modified flare-up protocol and 31 were performed with the mini-dose long protocol. Significantly more oocytes were obtained with the modified long protocol than the modified flare-up protocol. The number and quality of embryos available for transfer were similar in both groups. One clinical pregnancy (3.4%) was achieved with the modified flare-up protocol, and seven pregnancies (22.5%) were achieved using the mini-dose long protocol.

Ovarian cyst formation is a common complication of the long GnRHa protocol. It has been suggested as being typical for poor responders and as being a reliable predictor of poor stimulation and low pregnancy rates in a given cycle [87, 88]. Although the pathophysiology of ovarian cyst formation following GnRHa administration has not been completely elucidated, the higher the serum progesterone level at the time of commencing GnRHa administration, the lower the incidence of cyst formation [89]. Progestogen pre-treatment directly inhibits endogenous gonadotropin secretion and influences the pattern of gonadotropin and hypothalamic GnRH secretion. Three RCTs have demonstrated the successful use of progestins to prevent ovarian cyst formation during pituitary suppression in IVF cycles [90–92]. We have also successfully included progestin pre-treatment in the long mini-dose protocol [86].

It has to be recognized that the aforementioned studies varied in their definitions of POR, and as they were conducted well before the ESHRE consensus definition, none of them fulfilled the Bologna criteria for POR. An RCT comparing the efficacy of the long GnRHa protocol versus the short GnRHa protocol versus the GnRH-antagonist (GnRH-ant) protocol among women with a previous POR demonstrated the long GnRHa and the GnRH-ant protocols to be superior in terms of the numbers of oocytes retrieved. Women who had the short GnRHa protocol had significantly lower numbers of retrieved oocytes (2.71 ± 1.60) compared to the long protocol (4.42 ± 3.06) [93]. This study used stringent inclusion criteria, and POR was defined as a previous cancelled IVF cycle or three or fewer oocytes retrieved following stimulation with gonadotrophin ≥ 300 IU/day. Summarizing the preceding evidence, the long GnRHa protocol seems to be a suitable option for poor responders (see Figure 52.3).

GnRHa “stop” protocols

Pituitary recovery and resumption of gonadotropin secretion following GnRHa treatment may take up to several weeks, depending on the dose and route of administration of the agonist. For example, with intranasal buserelin acetate (BA), suppression of endogenous gonadotropin secretion seems to continue for at least 12 days after the discontinuation of the agonist [94], as was also reported for SC BA [95]. Interestingly, using the “ultrashort protocol,” suppression of endogenous LH secretion was more profound when LA administration was stopped after five days of administration, compared with continuous LA administration, and no premature LH peak was recorded [96]. This forms the basis for a variety of discontinuous or “stop” GnRHa protocols.

The preceding observations prompted several studies in which GnRHa were administered in the long protocol, but agonist administration was withheld once gonadotropin stimulation had started [97–101]. The majority of studies have shown favourable results in terms of both clinical outcome and cost-effectiveness,

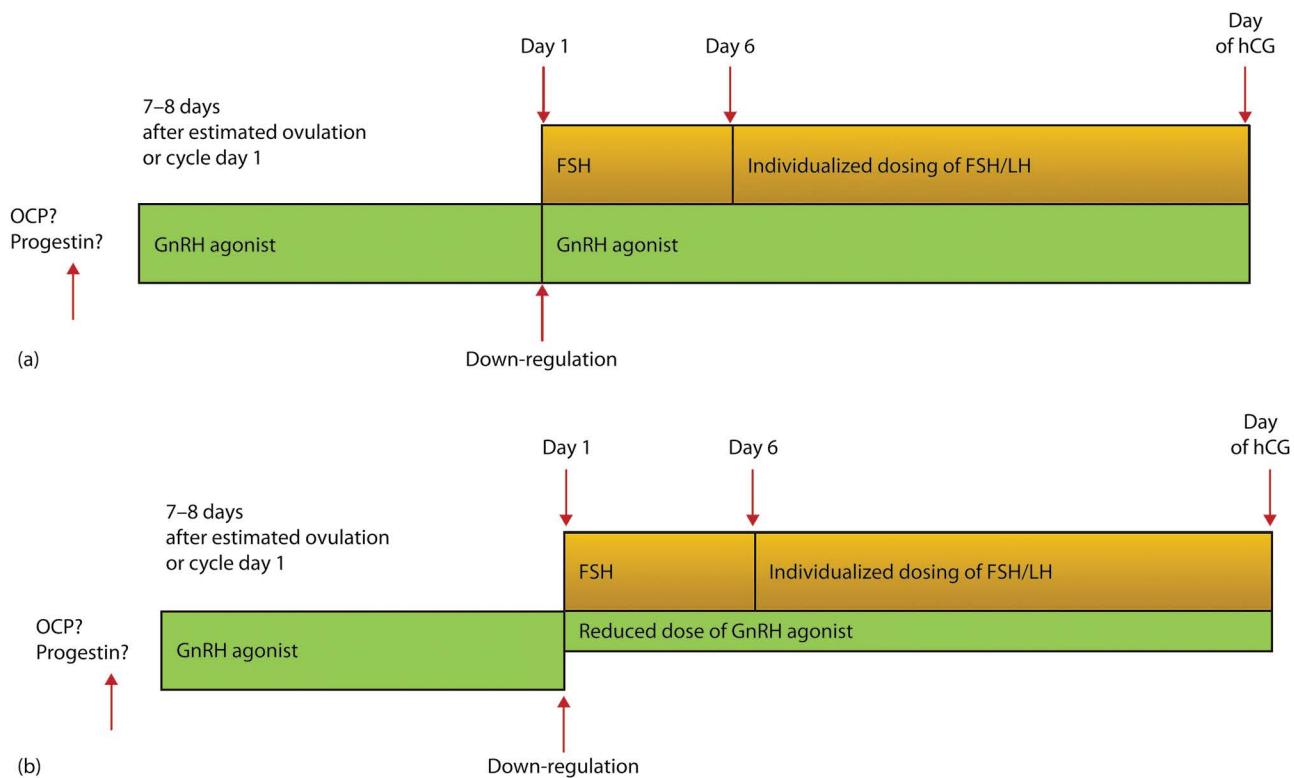


FIGURE 52.3 (a) The long GnRH agonist protocol. (b) The “mini-dose” long agonist protocol. Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; OCP, oral contraceptive pill.

but studies showing discouraging results were also reported [101]. Corson et al. [97] prospectively evaluated the effect of stopping GnRH_a (SC LA) therapy upon initiation of ovarian stimulation versus simultaneous GnRH_a and gonadotropin therapy. Both groups were found to be comparable in terms of the duration of stimulation and amount of exogenous gonadotropins required, as well as for any other stimulation or outcome parameter studied. Stopping LA upon initiation of ovarian stimulation did not reduce its efficacy at suppressing LH secretion, as in neither group was a premature LH surge detected.

Similar results were obtained in a prospective study that compared two protocols with variable duration of BA administration in an IVF/gamete intrafallopian transfer program [98]. No spontaneous premature LH surges were recorded in any of the groups, and all parameters of ovarian response to stimulation were found to be comparable for both groups. A trend towards a higher pregnancy rate per ET was noted in the discontinuous BA arm. Simons et al. [99] compared the efficacy of two early cessation protocols of triptorelin treatment with the conventional long protocol in IVF. In a multicentre RCT, 178 women were randomized to one of three treatment groups at the start of stimulation. SC triptorelin was started at the mid-luteal phase of the previous cycle and continued until the first day of gonadotropin treatment, or up to and including the fourth day of gonadotropin treatment or until the day of hCG injection. One premature LH surge was observed in the second group. Both early cessation protocols were at least as effective as the standard long protocol with regard to the number of oocytes, number of embryos, and ongoing pregnancy rate. It was concluded that early cessation of triptorelin on day 1 of gonadotropin treatment is as effective as the traditional

long protocol at preventing a premature LH surge and results in similar reproductive outcomes.

In contrast, Fujii et al. [101] reported on an RCT where 900 µg/day of intranasal BA was administered from the mid-luteal phase of the previous cycle until cycle day 7, when normal-responding patients were randomized to receive either gonadotropin stimulation alone or combined BA and gonadotropin therapy. The duration and total dose of gonadotropins administered were significantly increased in the early GnRH_a cessation group compared with the conventional long protocol. The numbers of fertilized oocytes and embryos transferred were significantly lower and the cancellation rate and rate of failed oocyte retrieval were significantly higher in the discontinuous long protocol. Although premature LH surges were not recorded in either group, serum progesterone and LH concentrations were significantly increased on the day of hCG administration with the discontinuous long protocol. Clinical pregnancy rates per transfer were similar for both protocols. It was concluded that early discontinuation of the GnRH_a is not beneficial and not cost-effective because of its adverse effects on follicular development and increased exogenous gonadotropin requirements, respectively. A reason for this could be because stopping daily agonist administration combined with ovarian stimulation leads to a further reduction in circulating LH concentrations [102], which supports the concept that there is still a small release of LH following daily agonist administration.

Discontinuous protocols were considered to be potentially beneficial for poor-responder patients undergoing IVF-ET [103]. Several trials with contradictory results have been reported. Faber et al. [104] conducted a single-group uncontrolled study in which poor-responder patients were treated with LA 0.5 mg/day

starting at the mid-luteal phase of the previous cycle. With the onset of menses, LA was discontinued and high-dose gonadotropin therapy was initiated. The cancellation rate was 12.5% (28/224 cycles), and only one case of premature LH surge was observed. Despite the uncontrolled nature of the study, a clinical pregnancy rate per transfer of 32% and an ongoing pregnancy rate per transfer of 23%, which seemed highly favourable for the specific subgroup of poor-responder patients, were achieved.

Subsequently, Wang et al. [105] conducted a prospective non-randomized study to determine the efficacy of a "stop" protocol in previously poor responders to a standard long protocol. Fifty patients were scheduled for 52 cycles of the modified "stop" agonist protocol. All patients received GnRHa from the mid-luteal phase of the previous cycle to the onset of menstruation, followed by high-dose gonadotropin stimulation. Six of the 52 cycles (11.8%) were cancelled because of POR. One premature ovulation was noted, and in the other 45 cycles, an average of 6.3 mature oocytes were retrieved. A favourable embryo implantation rate (11.5%) and clinical pregnancy rate (20.5%) were noted.

In a prospective study with historical controls involving 36 poor responders, the use of intranasal nafarelin (600 µg/day), commenced in the mid-luteal phase and discontinued on day 5 of ovarian stimulation, was evaluated [106]. The cancellation rate was 8.3%, and there was a trend towards increased peak E2 levels and an increase in the number of oocytes retrieved. The ongoing pregnancy rate per ET was 15%. A significant improvement in both the number and the quality of cleaving embryos was observed, and it was suggested therefore that discontinuation of the GnRHa leads to improved oocyte quality.

In another prospective study with historical controls [107], 39 "stop" nafarelin cycles in 30 previously poor-responder patients were compared to 60 past cycles in the same individuals. A significantly higher number of oocytes were retrieved and a higher number of embryos were available for transfer. No cases of premature LH surge were recorded. Pregnancy rates per ET and per cycle were 10.4% and 7.7%, respectively.

In contrast, Dirnfeld et al. [108] reported on an RCT involving 78 cycles in which a "stop agonist" regimen was compared with a standard long luteal protocol. Intranasal BA (1 mg/day) or SC triptorelin (100 µg/day) were initiated on day 21 of the previous cycle and ceased once pituitary suppression was confirmed. Ovarian stimulation was induced with the use of 225–375 IU/day hMG or purified FSH, commencing on the day of downregulation. A significantly higher cancellation rate was noted with the stop regimen compared with the controls (22.5% vs 5.0%, respectively). The stop and long regimens resulted in similar stimulation characteristics and clinical pregnancy rates (11% vs 10.3%, respectively). Only in patients with a basal FSH level that was not persistently high did the stop regimen result in a significantly higher number of retrieved oocytes compared with the standard long protocol (7.6 vs 4.0, respectively). It was concluded that, for most poor responders, the stop regimen offers no further advantage over the standard long protocol.

Garcia-Velasco et al. [109] designed an RCT in order to evaluate whether early cessation of the GnRHa (LA) is more beneficial than just increasing the doses of gonadotropins in poor-responder patients. Seventy poor-responder patients with normal basal FSH concentrations and a previous cancelled IVF cycle were randomly allocated to either a standard long protocol or a stop protocol. A significantly higher number of mature oocytes were obtained with the stop protocol compared with the standard long protocol (8.7 vs 6.2). The stop protocol significantly reduced the

gonadotropin requirements. Both protocols resulted in a similar cancellation rate (2.7% vs 5.8%), pregnancy rate (14.3% vs 18.7%), and implantation rate (12.1% vs 8.8%). It was concluded that the stop protocol combined with high doses of gonadotropins permitted the retrieval of a significantly higher number of oocytes but did not influence the reproductive outcome.

Recently, Orvieto et al. have suggested the combination of a GnRHa stop protocol with subsequent administration of GnRH antagonist in a flexible manner [110, 111]. A retrospective "proof of concept" study included 30 poor responders defined according to the Bologna criteria. The Stop GnRHa combined with multiple-dose GnRH antagonist revealed significantly higher numbers of follicles >13 mm on the day of hCG administration, higher numbers of oocytes retrieved, and top-quality embryos (TQE) with an acceptable clinical pregnancy rate (16.6%). Further studies are needed to evaluate this intervention.

Short GnRHa regimens

The short protocol consists of early follicular-phase initiation of GnRHa, with minimal delay before commencing gonadotropin ovarian stimulation. It takes advantage of the initial agonistic stimulatory effect of GnRHa on endogenous FSH and LH secretion, also known as the flare-up effect. In theory, it eliminates excessive ovarian suppression associated with prolonged agonist use. The duration of the endogenous gonadotropin flare has not been completely characterized, but pituitary desensitization is generally achieved within five days of initiating treatment [112], and therefore patients are protected from premature LH surges by the end of the stimulation phase. The short protocol has been proposed by many authors as a better stimulation protocol for poor responders [113–115].

In an early prospective study with historical controls and using an ultrashort protocol, Howles et al. [115] treated seven patients who had previously responded poorly to stimulation with clomiphene citrate (CC) and hMG with 0.5 mg/day BA during only the first three days of the cycle (ultrashort protocol). All seven patients had oocytes recovered and embryos replaced, and three out of these seven conceived (42.9%). Similarly, Katayama et al. [116] reported improved cycle outcomes in seven prior poor-responder patients with the short regimen. Garcia et al. [114] conducted a non-randomized prospective trial comparing long luteal and short flare-up agonist initiation in 189 cycles. They noted a significant decrease in exogenous gonadotropin requirements, higher pregnancy rates, and decreased miscarriage rates in patients receiving the flare-up regimen. In a retrospective comparison, Toth et al. [117] also reported that pregnancy and implantation rates were significantly higher and cancellation rates lower in patients with basal serum FSH levels ≥15 mIU/mL undergoing a flare-up regimen versus a long luteal agonist regimen. In a prospective uncontrolled study, Padilla et al. [113] administered a flare-up protocol with high-dose gonadotropins to 53 patients who were thought to be at risk for poor response after a "leuprolide acetate screening test." The cancellation rate was higher in poor flare-up LA test responders (11.3%) compared with good flare-up LA responders (1.1%) and luteal-phase long protocol cycles (1.8%). Despite a low number of oocytes retrieved, the ongoing pregnancy rate was 29% per retrieval and was considered favourable for this group of potentially poor-responder patients.

Despite these encouraging findings, other authors failed to confirm any substantial benefit of using a classic flare-up protocol. In a prospective study with historical controls [118], 80 poor responders were treated using a classic flare-up regimen with LA

0.5 mg/day from cycle day 2 and high-dose hMG from cycle day 3. Although the number of retrieved oocytes was increased (10 ± 6.6), the cancellation rate was high (23.4%), and the ongoing pregnancy rates of 6.5% per retrieval and 7.6% per transfer were disappointing. Brzyski et al. [119] reported that not only did concomitant initiation of GnRHa with purified urinary FSH result in poorer cycle outcome, but also an increased number of atretic oocytes were retrieved. A significant increase in LH and progesterone levels during the follicular phase was noted. Other groups using this approach also reported failure to improve ovarian response or cycle outcome in generally similar patient populations [120–122].

Despite the rationale for use of the short agonist protocol, the RCT comparing the long agonist versus the short agonist versus the antagonist protocols showed that the short agonist protocol was less effective than the long agonist protocol for poor responders [93]. In an RCT, San Roman et al. [123] have shown that a combination of early follicular-phase LA administration and hMG stimulation was associated with a significant increase in serum LH levels beginning with the first follicular-phase agonist dose, and with significant increases in serum progesterone and T levels during the follicular phase compared with midluteal GnRHa administration. The live birth rate/retrieval for the long protocol was 25% compared with 3.8% in the flare-up group. This may be the result of the initial flare-up effect of GnRHa on LH secretion causing raised LH levels. Evidence of an adverse effect of high endogenous LH levels during the follicular phase has led to the establishment of the ceiling theory [124]. According to this theory, beyond a certain ceiling level, LH suppresses GC proliferation and initiates atresia of less mature follicles.

Further support for this view comes from a study of Gelety et al. [125], who performed a prospective randomized crossover study of five regularly cycling women in order to determine the short-term pituitary and ovarian effects of GnRHa administered during differing phases of the menstrual cycle in the absence of gonadotropin stimulation. Each patient was administered LA 1 mg/day SC for five days beginning on cycle day 3, eight days post-LH surge, and 13 days post-LH surge with an intervening “washout” month. Significant increases in serum LH, E2, estrone, androgens, and progesterone levels were noted in the early follicular-phase group compared with the mid-luteal group. Early follicular initiation of the agonist resulted in a more pronounced suppression of FSH. It was suggested that relative FSH suppression and marked LH elevations could have potential detrimental effects on oocytes of the developing cohort that are often observed with flare-up regimens.

Can the adverse effects of the gonadotropin flare be prevented without losing the potential benefits of the short protocol? Two possible solutions have been suggested: the first is pre-treatment with an oral contraceptive pill (OCP) or a progestin. Cédrin-Durnerin et al. [126] noted that pre-treatment with a 12- to 20-day course of the progestin norethisterone before initiation of a flare-up regimen effectively lowered LH and progesterone levels during the early stages of gonadotropin stimulation. Many clinicians thus regard pre-treatment with an OCP or a progestin as integral in flare-up regimens, although this issue also became a matter of controversy [127]. The second solution is dose reduction of the GnRHa causing the flare, which forms the basis for “microdose flare” regimens (Figure 52.4).

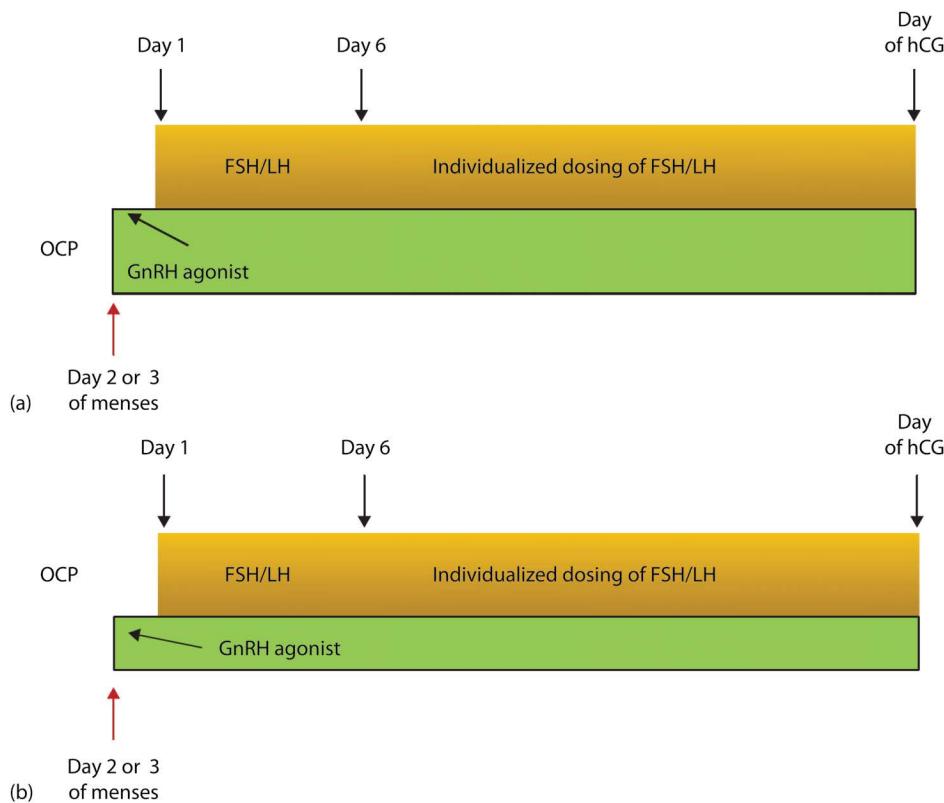


FIGURE 52.4 (a) The short GnRH agonist protocol. (b) The “microdose” flare GnRH agonist protocol. Abbreviations: FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; OCP, oral contraceptive pill.

Microdose flare GnRHa regimens

In theory, microdose flare regimens decrease the enhanced LH and progesterone secretion associated with standard flare-up regimens, as described earlier. Bstandig et al. [128] studied the hormonal profiles during the flare-up period using 25 and 100 µg of triptorelin in the short protocol. No significant difference in the magnitude of FSH and E2 release was observed between the two groups, but the maximal plasma LH level was significantly reduced after injection of 25 µg of triptorelin. It was suggested that in the flare-up protocol, a lower dose of GnRHa induces a hormonal flare-up that is more conducive to optimal follicular recruitment. Deaton et al. [129] have demonstrated that an extremely low dose of LA (25 or 50 µg) is needed to cause a pituitary flare of gonadotropins. Following a flare from 25 µg of LA on cycle day 2, the pituitary is able to recover and respond with a repeat flare on cycle day 5. These observations support the rationale behind the so-called microdose flare protocols.

Navot et al. [130] studied the effect of very low doses of GnRHa in cynomolgus monkeys and humans and established that 10 mg of histrelin in four divided doses (microdoses) could induce ovarian hyperstimulation in humans. Scott et al. [131] reported that an increase in gonadotropin levels could be induced in baboons with LA doses as low as 0.017 mg/kg. Although the minimal and optimal effective dose of GnRHa that can be successfully used to induce a gonadotropin flare in humans has not been thoroughly evaluated, several investigators have reported an improved outcome with doses as low as 20–40 µg of LA twice daily in poor responders.

In a prospective study with historical controls, Scott and Navot [132] treated 34 poor-responder patients with an OCP followed by 20 µg LA twice daily beginning on cycle day 3 and supplemented with exogenous gonadotropins beginning on cycle day 5. Ovarian responsiveness was enhanced with the microdose GnRHa stimulation cycle when compared with previous stimulation cycles. Specifically, the patients had a more rapid rise in E2 levels, much higher peak E2 levels, the development of more mature follicles, and the recovery of larger numbers of mature oocytes. None of the patients had a premature LH surge.

Impressive results using the microdose flare protocol were also reported in a prospective study with historical controls by Schoolcraft et al. [133]. Thirty-two patients, whose prior long luteal agonist cycles had been cancelled because of poor response, were now pre-treated with an OCP followed by follicular-phase administration of 40 µg LA twice daily beginning on cycle day 3 and high-dose FSH supplemented with human growth hormone (hGH) beginning on cycle day 5. Compared with the prior long luteal GnRHa cycle, there was a higher E2 response, more oocytes retrieved (10.9 per patient), fewer cycle cancellations (12.5%), and no premature LH surge or luteinization. For patients who were not cancelled, a favourable ongoing pregnancy rate of 50% was achieved.

In a prospective non-randomized trial with historical controls, Surrey et al. [134] treated 34 patients with a prior poor response to a standard mid-luteal long protocol with an OCP followed by LA 40 µg twice daily and high-dose gonadotropins. Cycle cancellation rates were dramatically reduced, and the mean maximal serum E2 levels obtained were significantly higher. The ongoing pregnancy rates per ET were 33% in patients aged ≤39 years and 18.2% in patients aged >39 years. Significant increases in circulating FSH levels occurred after five days of gonadotropin stimulation. No abnormal rises in LH, progesterone, or T during the follicular phase were noted. This could result from either the

lower GnRHa dose, the OCP pre-treatment, or a combination of the two.

Detti et al. reported on a retrospective cohort study that assessed the efficacy of three different GnRHa stimulation regimens to improve ovarian response in poor responders [103]. Women diagnosed as poor responders underwent three different stimulation regimens during IVF cycles:

Stop protocol: LA 500 µg/day administered from the mid-luteal phase to the start of menses, then gonadotropins from day 2 of the cycle. *Microdose flare:* LA 20 µg administered twice daily with gonadotropins from day 2 to the day of hCG administration. *Regular dose flare:* gonadotropins beginning with LA on day 2 at 1 mg/day for three days, followed by 250 LA µg/day until the day of hCG administration.

Since only 61 cycles were included in the analysis, none of the comparisons reached statistical significance; however, the microdose flare group demonstrated a trend towards a higher delivery rate.

It is noteworthy that, in a general IVF population (excluding poor responders), retrospective analysis failed to find the microdose flare protocol to be superior over the long mid-luteal agonist regimen [135]. Significantly higher cancellation rates (22.5% vs 8.2%), lower clinical pregnancy rates (47.3% vs 60%, non-significant), and a decreased number of oocytes retrieved per cycle (13.3 vs 16.5, non-significant) were noted with the microdose flare-up regimen.

Overall, all studies evaluating the microdose flare protocol were retrospective in nature. Obviously, large prospective RCTs are needed to validate the true efficacy of the microdose flare-up GnRHa regimens in poor responder patients.

GnRH antagonists in the treatment of poor responders

GnRH-ants competitively block the GnRH receptor in the pituitary gland, producing an immediate dose-related suppression of gonadotropin release. Within six hours of GnRH-ant administration, LH levels are significantly reduced. On the principle of maximizing potential endogenous pituitary stimulation, a GnRH-ant can be administered later in the follicular phase to suppress the LH surge [136, 137], thus avoiding suppression during the phase of early follicular recruitment (Figure 52.5). In the general IVF population, the GnRH-ants offer comparable therapeutic efficacy to agonists and have a number of potential advantages over

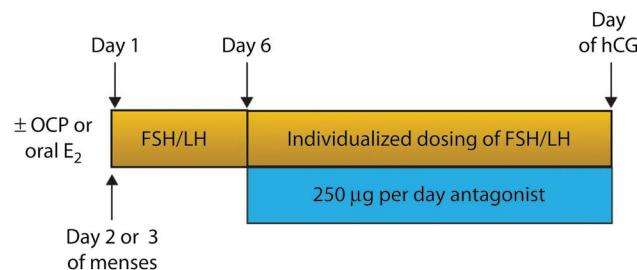


FIGURE 52.5 Gonadotropin-releasing hormone antagonist protocol. Abbreviations: E2, oestradiol; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; OCP, oral contraceptive pill.

agonists for use in ovarian stimulation protocols, such as avoiding the initial “flare-up” of LH, shortening the overall treatment period, reducing the risk of OHSS, and reducing menopausal side effects [136–138].

The GnRH-ants are administered in the late follicular phase, either according to the fixed or according to the flexible protocol (see Chapter 43). Thus, at the beginning of COS, the pituitary is fully susceptible to GnRH pulses. This may allow us to obtain a more natural follicular recruitment without any inhibitory effect possibly induced by the GnRHa. It has therefore been suggested as a suitable protocol for poor responders. GnRH-ants also permit the revival of stimulation protocols of the pre-agonist era using, for example, CC [139]. The combination of CC treatment in the early follicular phase and subsequent overlapping gonadotropin stimulation has been a standard therapy in the past [139, 140]. Owing to the synergistic effect of these compounds, the amount of gonadotropins required is lower and so are the costs [141, 142]. In addition, the gonadotropins counteract the detrimental effects of CC on the endometrium [141]. As a result of the high rate of premature LH surges, and therefore the high cancellation rate, this stimulation regimen was abandoned when GnRHa were introduced in IVF.

Craft et al. [143] were the first to suggest the use of GnRH-ants for COS in poor responders. In a small retrospective series, 18 previously poor responders were stimulated with a combination of gonadotropins and CC, and started on a GnRH-ant according to the flexible protocol. Compared to their poor response in a previous GnRHa cycle, modest improvements in cycle cancellation rates (29% vs 57%), oocyte yield (6.4 vs 4.7), and gonadotropin requirements (4506 vs 5468 IU) were noted with the GnRH-ant. Two live births resulted (11.8%). Several studies were subsequently undertaken in order to examine the efficacy of GnRH-ants in COS regimens designed for poor responders. The majority of these studies were of a small scale and retrospective. Retrospective studies will be presented first, followed by more recently reported RCTs.

Retrospective studies

Nikolettos et al. [144] compared 21 poor responders who underwent IVF–ICSI and were treated with a GnRH-ant protocol with 21 matched poor responders treated according to the long GnRHa protocol. Fifteen patients of the GnRH-ant group were treated with the combination of CC plus gonadotropins, while six patients were treated with gonadotropins alone. The use of the GnRH-ant protocol resulted in a significantly shorter treatment duration and lower gonadotropin consumption as compared with the use of the long GnRHa protocol. Three pregnancies (14.3%) were achieved with the antagonist and two (9.5%) with the long agonist protocol (non-significant).

Several retrospective studies have compared the GnRH-ant protocol with GnRHa flare-up and microdose flare regimens. In a retrospective cohort study, Posada et al. [145] compared the clinical outcome of COS in unselected patients undergoing IVF with a GnRH-ant (133 cycles) versus a four-day ultrashort GnRHa regimen (236 cycles). The GnRH-ant protocol was shown to reduce treatment duration and amount of gonadotropin used. In younger women, the antagonist protocol was associated with significantly better pregnancy and implantation rates, but no difference was observed in pregnancy rates in patients aged >38 years.

Mohamed et al. [146] retrospectively compared the agonist flare-up and antagonist protocols in the management of poor responders to the standard long protocol. A total of 134 patients

undergoing IVF–ICSI treatment who responded poorly to the standard long protocol in their first treatment cycle were studied. In the second cycle, 77 patients received a short GnRHa flare-up regimen and 57 patients received an antagonist protocol, based solely on physician preference. There were no cycle cancellations in the flare-up protocol and there was a 7% cancellation rate in the antagonist protocol due to lack of response. A significantly higher number of patients had ET in the flare-up protocol. Similar numbers of oocytes (5.4 vs 5.2) and similar implantation and pregnancy rates per cycle (12.8% and 17.5% vs 12.8% and 24.7%) were reported in the antagonist and flare-up groups, respectively. It was concluded that both the flare-up and the antagonist protocols significantly improved the ovarian response of previously poor responders. However, a significantly higher cycle cancellation rate and fewer patients having ET in the antagonist group suggested a higher efficacy for the flare-up regimen.

Conflicting results were reported by Fasouliotis et al. [147], who also conducted a retrospective analysis between the flare-up and antagonist regimens in poor responders. Of 56 poor responders treated with the flare-up protocol, 53 who failed to conceive were subsequently treated in the next cycle with a GnRH-ant regimen. While ovarian response did not differ between the two protocols, the number of embryos transferred was significantly higher in the GnRH-ant group (2.5 ± 1.6 vs 2.0 ± 1.4 , respectively). The clinical pregnancy and implantation rates per transfer in the GnRH-ant group tended to be higher than in the flare-up group but did not reach significance (26.1% and 10.7% compared with 12.2% and 5.9%, respectively). The ongoing pregnancy rate per transfer was significantly higher in the GnRH-ant than in the GnRHa flare-up group (23.9% vs 7.3%, respectively).

Copperman [148] conducted a retrospective analysis with historic controls comparing cycle outcomes in poor responders who had stimulation protocols that included an antagonist with those with the microdose flare protocol. Patients were placed in the antagonist or microdose flare treatment groups usually after failing in an LA downregulation cycle, and often according to physician preference. The results of this retrospective analysis indicated that, for poor responders, the inclusion of a GnRH-ant in the treatment regimen significantly increased clinical pregnancy rates and significantly lowered cancellation rates compared with patients treated with the microdose flare protocol.

The use of OCP pre-treatment in antagonist cycles for poor responder patients is also of clinical relevance, as their ovarian reserve may be especially sensitive to suppression of endogenous gonadotropins by the pill. Copperman [148] reported a retrospective study of 1343 patients, where poor responders were given a starting dose of 450 IU of gonadotropin. In the OCP pre-treatment group, patients were administered OCP for 18–24 days, beginning on cycle day 3. Patients were first administered a combination of r-FSH and hMG on cycle day 3, and were administered GnRH-ant when their lead follicle reached 14 mm. An additional 75 IU of hMG was administered beginning on the first day of antagonist treatment. Patients whose antagonist stimulation cycle included OCP pre-treatment had a significantly higher pregnancy rate and a significantly lower cancellation rate. In addition, a higher proportion of patients obtained more than eight oocytes following OCP pre-treatment. In contrast, Shapiro et al. [149] reported significantly increased cancellation rates (23%) in a group of poor-responder patients pre-treated with an OCP compared with patients not receiving OCP pre-treatment (9%). The two studies,

however, differed both in inclusion criteria and in the use of LH in the stimulation protocol.

Prospective studies

Akman et al. [150] compared a GnRH-ant protocol to a protocol using gonadotropins alone in poor responders. In total, 20 women were randomized to each group. Women assigned to the antagonist arm received 0.25 mg of cetrorelix according to the flexible protocol, and all women were initially stimulated with 600 IU of urinary-derived gonadotropin. There was no statistically significant difference between the groups for cancellation rates, gonadotropin requirements, number of mature oocytes retrieved, E2 concentrations on the day of hCG administration, fertilization rates, and number of embryos transferred. The clinical pregnancy and implantation rates in the antagonist group appeared higher but were not significantly different (20.00% and 13.33% compared with 6.25% and 3.44%, respectively) because of the small numbers involved.

There are several RCTs that compare the agonist flare-up with the antagonist protocols. Akman et al. [151] compared clinical outcomes of 48 poor-responder patients who were treated with either a microdose flare (LA 40 µg SC per day) protocol or the antagonist (cetrorelix 0.25 mg daily) protocol. All patients received 300 IU of highly purified FSH and 300 IU of hMG for four days, followed by individual adjustments in the dose of highly purified FSH. Patients in the microdose flare group also received OCP pre-treatment. There was no difference in the median total treatment doses of gonadotropins between the two groups. Serum E2 levels on the day of hCG administration and the number of oocytes retrieved were significantly lower in the antagonist group. No differences were observed between the two groups for fertilization rates, number of embryos transferred, and, most importantly, implantation rates and ongoing pregnancy rates per transfer. It was concluded that the efficacy of these stimulation protocols in poor-responder patients was comparable, but larger studies were needed.

De Placido et al. [152] randomized 133 women “at risk for poor ovarian response” to undergo COS by either a modified GnRH-ant protocol or a short flare-up regimen. Patients in the antagonist arm were treated by the flexible regimen with 300 IU of r-FSH given from cycle day 2. When the lead follicle reached a diameter of 14 mm, cetrorelix 0.125 mg was given daily for two days followed by cetrorelix 0.25 mg daily until the day of hCG administration. Beginning on the same day of GnRH-ant administration, a daily dose of 150 IU of r-LH (Luveris) was also added until the day of hCG administration. Patients in the flare-up arm received a daily dose of triptorelin (0.1 mg SC), beginning on the same day of the first r-FSH administration. In addition, in this group, a dose of 150 IU/day of r-LH was added when at least one follicle reached 14 mm. The mean number of metaphase II oocytes (primary endpoint) was significantly higher in the antagonist group (5.73 ± 3.57 vs 4.64 ± 2.23 , respectively; $p < 0.05$). Cancellation rates, gonadotropin requirements, implantation rates, and clinical and ongoing pregnancy rates were all comparable for the two groups.

Demirogl and Gurgan [153] conducted an RCT comparing the short microflare and the flexible GnRH-ant protocols in 90 poor-responder patients. In the microflare group, 45 patients received an OCP and, on the third day of menstruation, 40 µg SC twice daily of LA followed by 450 IU/day of hMG. In the antagonist group, 45 patients received 450 IU/day hMG starting on day 3 and 0.25 mg cetrorelix administered daily when two or more follicles

reached 13–14 mm in diameter. The total gonadotropin dose used was significantly higher in the antagonist group, while the number of oocytes retrieved was significantly greater in the microflare group (4.3 ± 2.13 vs 3.1 ± 1.09 ; $p = 0.001$). The implantation rate was significantly higher in the microflare group than in the antagonist group (22% vs 11%; $p = 0.017$). It was concluded that the short microflare protocol seems to have a better outcome in poor-responder patients, with a significantly higher mean number of mature oocytes retrieved and a higher implantation rate.

Kahraman et al. [154] conducted another RCT comparing the microflare and the antagonist protocols in patients who previously had a low response to the long GnRH-ant protocol. Twenty-one patients received LA (50 µg twice daily) starting on the second day of post-OCP bleeding. The other 21 patients received 0.25 mg of cetrorelix daily when the leading follicle reached 14 mm in diameter. Stimulation in both groups consisted of 300–450 IU daily doses of r-FSH. The mean serum E2 concentration on the day of hCG administration was significantly higher in the microflare group than in the antagonist group (1904 vs 1362 pg/mL; $p = 0.042$), but all other outcome variables studied were found to be comparable for the two groups. It was concluded that the microflare agonist and multiple dose GnRH-ant protocol have similar efficacy in terms of improving treatment outcomes of poor-responder patients. Very similar findings were reported by Devesa et al. [155], who compared the microflare agonist and multiple-dose antagonist protocols in 221 poor-prognosis patients based on previous cycles or clinical criteria. Except for significantly higher serum E2 levels on hCG administration day in the microflare group, all other outcome variables were found to be comparable for the two groups.

Schmidt et al. [156] randomized 48 previously poor responder patients to either a GnRH-ant protocol (ganirelix 0.25 mg daily in a flexible manner) or a microdose flare regimen (LA, 40 µg twice daily, after OCP pre-treatment). Ovarian stimulation consisted of 300 IU of r-FSH every morning and 150 IU of hMG every evening. Cancellation rates due to an inadequate response were equally high, being close to 50% in both groups. While only 13 women in the antagonist group and 11 women who received a microdose flare completed their cycles, no significant differences in oocyte yield (8.9 vs 9), fertilization rate (69.1% vs 63.5%), or clinical pregnancy rate (38.5% vs 36.4%) were detected. It was concluded that the antagonist protocol appears to be as effective as the microdose flare protocol for COS in poor responders but could be a superior choice in terms of cost and convenience for the patient.

Malmusi et al. [157] compared the efficacy of the flare-up GnRH-ant protocol to the flexible GnRH-ant protocol in poor responders. Fifty-five poor-responder patients undergoing IVF–ICSI were randomized to receive either triptorelin (100 µg daily) from the first day of menstruation followed by exogenous gonadotropins from the second day of menstruation (30 cycles), or exogenous gonadotropins from the first day of menstrual cycle and later ganirelix (0.25 mg daily) once the leading follicle reached 14 mm in diameter (25 cycles). Gonadotropin requirements were significantly reduced with the flare-up protocol. The number of mature oocytes retrieved, fertilization rate, and top-quality embryos transferred were significantly increased in the flare-up compared to the GnRH-ant group. The implantation and pregnancy rates were similar in both groups.

Very few RCTs comparing the long GnRH-ant and the GnRH-ant for COS in poor responders have been published (Table 52.1) [158–161]. Studies vary and suffer from considerable heterogeneities in terms of almost all possible aspects, such as inclusion criteria,

TABLE 52.1 Cycle Characteristics of Randomized Controlled Trials Comparing the Long Gonadotropin-Releasing Hormone Agonist and Gonadotropin-Releasing Hormone Antagonist Protocols in Poor-Responder Patients Undergoing *In Vitro* Fertilization

Study	Inclusion Criteria	Number of Patients on Agonist	Number of Patients on Antagonist	Long Protocol	Antagonist Protocol	Gonadotropin Type and Dose	Cancellation Rate (%)	Stimulation Duration (Days)	Gonadotropin Consumption		Number of Oocytes Retrieved	Implantation Rate (%)	Clinical	Ongoing/
									Ampoules or FSH Units	Oocytes Retrieved			Pregnancy Rate Started Cycle (%)	
D'Amato et al. [158]	<3 oocytes retrieved in >2 long agonist cycles or cancelled cycles	60	85	Depot leuprolide, dose not given	Cetralelix, multi-dose, flexible	Agonist: r-FSH, individualized dose Antagonist: clomiphene 100 mg days 2–6 plus r-FSH 300 IU	34 (agonist) 4.8 (antagonist)	—	50.05 ± 5.11 (agonist)	3.36 ± 1.3 (agonist)	7.6 (agonist) 13.5 (antagonist)	15.3 (agonist) 22.2 (antagonist)	NA	
Cheung et al.[161]	<3 mature follicles on previous long protocol or basal FSH >10 IU/L	31	32	Luteal, nasal buserelin 60 µg daily following OCP	Cetralelix, multi-dose, fixed (S6)	R-FSH 300 IU	34.4 (agonist) 38.7 (antagonist)	11.5 ± 2.4 (agonist) 10.5 ± 2.7 (antagonist)	3445 ± 730 (agonist) 3150 ± 813 (antagonist)	5.62 ± 4.17 (agonist) 5.89 ± 3.02 (antagonist)	13.3 (agonist) 13.6 (antagonist)	9.4 (agonist) 16.1 (antagonist)	NA	
Marci et al. [160]	<3 oocytes retrieved and E2 maximum <600 pg/mL on a previous standard long protocol	30	30	Luteal, depot leuprolide 3.75 mg	Cetralelix, multi-dose, flexible	R-FSH 375 IU	13.3 (agonist) 3.3 (antagonist)	14.6 ± 1.2 (agonist) 9.8 ± 0.8 (antagonist)	72.6 ± 6.8 (agonist) 49.3 ± 4.3 (antagonist)	4.3 ± 2.2 (agonist) 5.6 ± 1.6 (antagonist)	NA	6.6 (agonist) 16.6 (antagonist)	0 ag 13.3 16.6 (antagonist)	
Tazegul et al. [159]	FSH <13 mIU/mL, E2 maximum <500 pg/mL on hCG day, <3 mature follicles, <4 oocytes retrieved	45	44	Luteal leuprolide 1 mg, decreased to 0.5 mg upon downregulation	Cetralelix/ ganirelix, multi-dose, flexible	R-FSH 300 IU and 300 IU hMG	6.8 (agonist) 9.0 (antagonist)	12.03 ± 2.86 (agonist) 10.6 ± 1.63 (antagonist)	3872.7 ± 1257.1 (agonist) 2467.7 ± 342.4 (antagonist)	5.47 ± 2.45 (agonist) 5.44 ± 1.29 (antagonist)	NA	24.4 (agonist) 22.7 (antagonist)	22.2 ag 18.1 (antagonist)	

Abbreviations: FSH, follicle-stimulating hormone; r-FSH, recombinant follicle-stimulating hormone; E2, oestradiol; OCP, oral contraceptive pill; S6, stimulation day 6; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin.

agonist and antagonist administration regimens, and outcome variables reported. For example, in two studies [158, 160], a depot preparation of a GnRHa was used, which is not a recommended administration route for low responders. In contrast, in the study by Tazegul et al. [159], a mini-dose agonist protocol was used, which is certainly a more appropriate administration route for low responders. Since these studies are not readily comparable, only general conclusions can be made. It appears that the GnRH-ant protocol is as effective as the long agonist protocol in poor responders. Gonadotropin consumption and stimulation duration both appear to be reduced with the antagonist protocol, a considerable practical advantage for patients. Clinical pregnancy and live birth rates appear to be similar.

Alternative approaches and treatment

protocols using GnRH-ant

One of the problems often seen in poor-responding patients is a shortened follicular phase, which limits the ability to recruit a sizable cohort of follicles. Frankfurter et al. [162] described a novel use of a GnRH-ant before ovarian stimulation in an attempt to lengthen the follicular phase, aiming to lengthen the recruitment phase of the cycle to allow for the rescue of more follicles once gonadotropin stimulation was initiated. Twelve patients who previously exhibited a poor response to a standard (long, short, or antagonist) protocol were included. According to this regimen, patients received two doses of 3 mg of cetrorelix (which is no longer commercially available), the first on cycle days 5–8 and the second four days later. With cetrorelix commencement, medroxyprogesterone acetate (MPA; 10 mg daily) was given and was continued until ovarian suppression was confirmed. Then, a combination of r-FSH (225 IU SC twice daily) and r-hCG (2.5 mg SC four times a day) was initiated, and MPA was discontinued to allow for vaginal bleeding. When a lead follicle size of 13 mm was observed, daily cetrorelix (0.25 mg SC) was started and continued until hCG triggering. By using a GnRH-ant in the follicular phase before ovarian stimulation, significant improvements in oocyte, zygote, and embryo yields were achieved. A trend towards improved implantation (21%), clinical pregnancy (41.7%), and ongoing pregnancy (25%) rates in the follicular GnRH-ant cycle was also noted. More prospective studies are needed in order to examine the efficacy of this novel therapeutic approach.

Orvieto et al. [110] described the combination of the microflare GnRHa protocol and a GnRH-ant protocol in poor responders. This protocol combines the benefits of the stimulatory effect of the microflare on endogenous FSH release with the immediate LH suppression induced by the GnRH-ant, and was therefore suggested as a valuable new tool for treating poor responders [110, 163]. The stimulation characteristics of 21 consecutive ultrashort GnRHa/GnRH-ant cycles in 21 patients were compared with their previous failed cycles [110]. Triptorelin (100 µg SC) was started on the first day of menses and continued for three consecutive days, followed by high-dose gonadotropins, which were initiated two days later. Once the lead follicle had reached a size of 14 mm and/or E2 levels exceeded 400 pg/mL, cetrorelix (0.25 mg/day) was introduced and continued up to and including the day of hCG administration. The number of follicles >14 mm on the day of hCG administration, the number of oocytes retrieved, and the number of embryos transferred were all significantly higher in the study protocol as compared with the historic control cycles. A reasonable clinical pregnancy rate (14.3%) was achieved.

Another innovative protocol using GnRHa/GnRH-ant conversion with oestrogen priming (AAZEP) in poor responders has

been reported by Fisch et al. [164] and is described later in this chapter (in the section entitled “Luteal-phase manipulations”).

NCs and modified NCs

The yield of lengthy, high-dose, and cost stimulation regimens used in poor responders to increase the number of oocytes retrieved is often disappointing. It was therefore suggested to perform NC-IVF in such cases, an approach that is less invasive and less costly for the patient.

Terminology

The International Society for Mild Approaches in Assisted Reproduction (ISMAAR) has recommended revised definitions and terminology for NC-IVF and different protocols used in ovarian stimulation for IVF [165]. This was the result of the broad inconsistencies existing in the terminology used for definitions and protocols for ovarian stimulation in IVF cycles, as will be seen later in this text. The term “natural cycle IVF” should be used when IVF is carried out with oocytes collected from a woman’s ovary or ovaries in a spontaneous menstrual cycle without administration of any medication at any time during the cycle. The aim of this cycle is to collect a naturally selected single oocyte at the lowest possible cost. The term “modified natural cycle” (MNC) should be applied when exogenous hormones or any drugs are used when IVF is being performed during a spontaneous cycle with the aim of collecting a naturally selected single oocyte but with a reduction in the chance of cycle cancellation. This could include the following scenarios: (i) the use of hCG to induce final oocyte maturation (luteal support may/may not be administered); and (ii) the administration of GnRH-ant to block the spontaneous LH surge with or without FSH or hMG as add-back therapy (an hCG injection and luteal support are administered).

The aforementioned terminology has not yet been well incorporated into clinical practice. In all of the following studies presented on NC-IVF, hCG was used for ovulation triggering, and the term MNC is used when a combination of GnRH-ant and gonadotropins is given. In a prospective study with historical controls, Bassil et al. [166] analysed 11 patients who underwent 16 NCs (with hCG administration) for IVF. These were compared with 25 previous failed cycles with poor response in the same patients. The cancellation rate in NCs was 18.8% compared with 48% in stimulated cycles. Three ongoing pregnancies were obtained in NCs (18.8% per started cycle) compared with none in stimulated cycles. In another prospective study with historical controls, Feldman et al. [167] compared 44 unstimulated IVF cycles in 22 poor-responder patients with those of 55 stimulated cycles of the same patients during the 12 months prior to the study. Eighteen (82%) patients had at least one oocyte retrieved, while nine (41%) had at least one cycle with ET. Two (9%) patients each gave birth to a healthy term baby. These results were comparable with those of the stimulated cycles. In a small retrospective study [168], 30 patients who had previously been cancelled because of POR underwent 35 NCs, achieving an ongoing pregnancy rate of 16.6% per oocyte retrieval and an implantation rate of 33%. All patients, however, were <40 years old and had a mean day-3 FSH of 11.1 IU/L.

Similar results were found in an observational study with no controls, in which patients aged 44–47 years were included [169]. These patients were recruited based on age only, without prior demonstration of poor response. Out of 48 treatment cycles conducted in 20 women, oocyte retrieval was successful in 22 cycles

(46%). Fertilization and cleavage rates of 48% and 100%, respectively, were obtained. One biochemical and one ongoing pregnancy were achieved. Thus, the ongoing pregnancy rate was 5% per patient and 2.08% per cycle.

Check et al. [170] reported on 259 retrieval cycles and 72 transfers in poor responders using minimal or no gonadotropin stimulation and without GnRHa or GnRH-ant. These patients were divided into four age groups (<35, 36–39, 40–42, and >43 years) and their mean serum day-3 FSH levels were 19.7, 20.6, 18.8, and 21.9 mIU/mL, respectively. In total, 12 deliveries were achieved after 259 IVF cycles (4.6%). Eliminating the oldest age group, the delivery rate for 47 ETs in women aged ≤42 years was 25.5%. Approximately 50% of retrievals resulted in an embryo (about half were transferred fresh and half frozen). The median number of embryos transferred was one. The implantation rate was 21.6% for the three groups, 33.3% for patients aged <35 years, and 28.6% for women aged 36–39 years. It was concluded that pregnancies and live births can be achieved in poor-prognosis/poor-responder patients with elevated basal FSH levels, and age was found to be a more adverse infertility factor than elevated serum FSH.

The only RCT on this topic [171] compared the efficacy of NC-IVF with the microdose GnRHa flare protocol in poor responders. A total of 129 patients who were poor responders in a previous IVF cycle were included: 59 women underwent 114 attempts of NC-IVF and 70 women underwent 101 attempts of IVF with COS by microdose agonist flare. In the NC patients, the oocyte retrieval procedure was performed in 114 cycles, and oocytes were found in 88 of these (77.2%). The poor responders treated with NC-IVF and those treated with microdose GnRHa flare showed similar pregnancy rates per cycle and per transfer (6.1% and 14.9% vs 6.9% and 10.1%, respectively). The women treated with NC-IVF showed a statistically significant higher implantation rate (14.9%) compared with controls (5.5%). When subdivided into three groups according to age (≤35 years, 36–39 years, and >40 years), younger patients had a better pregnancy rate than the other two groups. It was concluded that in poor responders, NC-IVF is at least as effective as COS, especially in younger patients, with a higher implantation rate.

Papaleo et al. [172] reported on a series of poor-prognosis patients, all of them with AMA, elevated serum FSH, and reduced AFC, who underwent NC-IVF. A total of 26 NCs in 18 patients were analysed. Pregnancy was achieved in three patients, of which two patients were ongoing (11.5% per cycle, 20.0% per ET). It was suggested that since the overall pregnancy rates achieved were comparable with those of conventional IVF-ET in poor responders, considering the lower costs and risks and the patient-friendly nature of such protocols, NC-IVF can provide an acceptable alternative option for persistent poor responders.

There have been recent studies addressing the efficacy of NC-IVF in the Bologna criteria poor responders. In a retrospective cohort study by Polyzos et al. [173], 164 consecutive patients undergoing 469 NC-IVF cycles were included, with 136 patients (390 cycles) fulfilling the Bologna criteria definition of POR and 28 women (79 cycles) considered as normal responders. The live birth rates per cycle were 2.6% versus 8.9% among Bologna criteria poor responders and normal responders and the live birth rates per treated patient were 7.8% versus 25%. The conclusion from this study was that Bologna criteria poor responders did not experience substantial benefits with NC-IVF.

Modified NC

The efficacy of NC-IVF is hampered by high cancellation rates because of premature LH rises and premature ovulations [174]. The possibility of enhancing the efficacy of unstimulated IVF cycles by the concomitant addition of a GnRH-ant and exogenous gonadotropins in the late follicular phase was introduced by Paulson et al. as early as 1994 [175]. This protocol, later known as the MNC, is expected to reduce the rate of premature ovulation and to improve control of gonadotropin delivery to the developing follicle.

In a preliminary report on 44 cycles in 33 young, normal-responder patients [176], the cancellation rate was 9%, and in 25% of retrievals, no oocyte was obtained. ET was performed in 50% of the started cycles, leading to a clinical pregnancy rate of 32.0% per transfer and 17.5% per retrieval, of which five (22.7% per transfer) were ongoing. It was suggested that the MNC could represent a first choice IVF treatment with none of the complications and risks of current COS protocols, a considerably lower cost, and an acceptable success rate.

Considerable experience with the MNC protocol in the general IVF population has been accumulated by the Dutch group in Groningen [177–179]. In a preliminary report, the cumulative ongoing pregnancy rate after three cycles with this protocol was 34% and the live birth rate per patient was 32% [179]. Summarizing a much larger experience, the same group [178] later reported on a total of 336 patients who completed 844 cycles (2.5 per patient). The overall ongoing pregnancy rate per started cycle was 8.3% and the cumulative ongoing pregnancy rate after up to three cycles was 20.8% per patient. In a recent report of further follow-up of up to nine cycles [172, 177], a total of 256 patients completed 1048 cycles (4.1 per patient). The ET rate was 36.5% per started cycle. The ongoing pregnancy rate was 7.9% per started cycle and 20.7% per ET. Including treatment-independent pregnancies, the observed clinical pregnancy rate after up to nine cycles was 44.4% (95% confidence interval [CI] 38.3%–50.5%) per patient. Pregnancy rates per started cycle did not decline in higher cycle numbers (overall 9.9%) but dropout rates were high (overall 47.8%).

Several studies have been reported on the use of the MNC protocol in poor responders. Kolibianakis et al. [180] evaluated the use of the MNC for IVF in poor responders with an extremely poor prognosis as a last resort prior to oocyte donation. Thirty-two patients with regular menstrual cycles, basal FSH levels >12 IU/L, and one or more failed IVF cycles with five or fewer oocytes retrieved were included. Recombinant hFSH 100 IU and ganirelix 0.25 mg/day were started concomitantly when a follicle with a mean diameter of 14 mm was identified. hCG was administered as soon as the mean follicular diameter was ≥16 mm. Twenty-five out of 78 cycles performed (32.1%) did not result in oocyte retrieval. In nine out of 53 cycles (16.9%) in which oocyte retrieval was performed, no oocytes were retrieved. ET was performed in 19 out of 44 cycles in which oocytes were retrieved (43.2%), but no ongoing pregnancy was achieved in 78 MNC cycles. It was concluded that the MNC does not offer a realistic chance of live birth in poor-prognosis/poor-responder patients when offered as a last resort prior to oocyte donation.

Studies with somewhat more encouraging outcomes were also reported. Elizur et al. [181] retrospectively evaluated 540 cycles in 433 poor responders who were divided by treatment protocol into MNC, GnRH-ant, and long agonist groups: there were 52 MNC cycles, 200 GnRH-ant cycles, and 288 long GnRHa cycles. In the MNC protocol, a GnRH-ant 0.25 mg/day and two to three

ampules of hMG were administered daily once the lead follicle reached a diameter of 13 mm. The mean number of oocytes retrieved in the MNC group was significantly lower than in the stimulated antagonist and long agonist groups (1.4 ± 0.5 vs 2.3 ± 1.1 and 2.5 ± 1.1 , respectively; $p < 0.05$). The respective implantation and pregnancy rates were comparable (10% and 14.3%, 6.75% and 10.2%, and 7.4% and 10.6%). The number of cancelled cycles was significantly higher in the MNC group. Cancellations due to premature luteinization or failure to respond to stimulation were significantly more common in patients aged >40 years. As pregnancy rates were comparable for all groups, it was concluded that the MNC is a reasonable alternative to COS in poor responders.

The only RCT on this issue was performed to investigate the value of MNC-IVF compared with the conventional GnRH-ant cycle in low responders [182]. The study population consisted of 90 patients with low response in previous cycles who had undergone 90 IVF cycles. Forty-five patients were randomly allocated into the MNC-IVF protocol and 45 into the GnRH-ant protocol. In the MNC arm, SC injections of 0.25 mg cetrorelix and 150 IU r-FSH were started concomitantly when the lead follicle reached 13–14 mm in diameter and were continued daily until the day of hCG administration. In the antagonist group, patients received a conventional, multiple-dose, flexible GnRH-ant protocol with 225 IU of r-FSH administered daily from cycle day 3. In the MNC group, 8 out of 45 cycles initiated (17.8%) had to be cancelled before ET because no oocytes were available. Four out of 45 cycles initiated (8.9%) did not result in oocyte retrieval owing to no follicular development or premature ovulation and no oocytes were found in 4 out of 41 cycles (9.8%) in which oocyte retrieval was performed. In the antagonist group, 3 out of 45 cycles initiated (6.7%) were cancelled before ET. Despite the difference in cancellation rate between the two groups, it was not statistically significant. The numbers of oocytes, mature oocytes, fertilized oocytes, grade 1 or 2 embryos, and embryos transferred were all significantly lower in the MNC group. Gonadotropin requirements and number of days of r-FSH required for COS were significantly fewer in the MNC group than in the antagonist group. Finally, clinical pregnancy rates per cycle initiated and per ET of the MNC group were similar to those of the antagonist group (13.3% and 17.8%; 16.2% and 19%, respectively). Live birth rates per ET and implantation rates were also comparable between the two groups (13.5% and 16.7%; 12.5% and 9.8%, respectively). It was concluded that the MNC provides comparable pregnancy rates to GnRH-ant-based COS with lower doses and shorter durations of FSH administration, and thus could be a patient friendly and cost-effective alternative in low responders.

In summary, the options of NC- or MNC-IVF are safe, patient-friendly treatments with low costs of medication, especially in those who are refractory to COS and decline the option of oocyte donation. Despite the advantages of this approach, its low efficiency has restricted its widespread use. Patients should be fully informed of the advantages and disadvantages of NC- or MNC-IVF protocols. From the preceding studies, it is evident that the likelihood of retrieving an oocyte is between 45% and 80%, the likelihood of reaching ET is around 50%, and the likelihood of pregnancy and live birth is between 0% and 20% (generally around 5%), depending largely on age and ovarian reserve. Younger patients with diminished ovarian reserve (DOR) have a much better prognosis [178, 183]. The use of indomethacin during the late follicular phase has been suggested in order to decrease the spontaneous ovulation rate and hence provide a higher oocyte retrieval success rate in MNC-IVF [183, 184].

The exact role of NC and MNC protocols in patients with DOR has yet to be determined, as are several key issues that have not yet been subjected to testing, such as:

1. Is the MNC protocol superior to the simple NC protocol? No study so far has evaluated these two regimens.
2. What is the best timing for hCG administration and what is the ideal time interval between hCG administration and egg retrieval? Different authors used different criteria for triggering ovulation. While many authors regard follicle size ≥ 16 mm as the threshold [171, 180, 185, 186], others prefer to administer hCG at 17–18 mm [182], or even ≥ 18 mm [168, 181]. Segawa et al. [187] prefer the use of GnRHa for ovulation triggering than hCG. While no consensus exists, the best estimate is that early ovulation triggering (i.e. ≥ 16 mm) is beneficial [188].
3. Are oocyte and embryo quality improved in NCs? While there is a common belief that “natural” is better, this assumption has never been directly tested.
4. How many attempts should be made? Schimberni et al. have reported fairly constant implantation and pregnancy rates through five NC cycles [185]. Castelo Branco et al. [186] have reported a cumulative pregnancy rate of 35.2% after three MNC cycles. The best estimate is that three to five cycles should be offered.
5. What is the role of follicle flushing? While in the general IVF population the use of follicle flushing was abandoned, there are studies suggesting that flushing may improve oocyte yield in poor responders [189–191]. Others [192], however, have failed to show any beneficial effect.
6. Should cleavage- or blastocyst-stage transfers be performed?
7. Which dose of gonadotropins should be administered in the MNC protocol? Different authors have used doses ranging from 100 IU r-FSH [180] or 150 IU [186] and up to 225 IU [181]. The optimal dose needed to support a single follicle in conjunction with GnRH-ant administration has not been determined.
8. Should LH be included in the gonadotropin regimen? In patients with POR, the addition of LH to the stimulation regimen might be beneficial [193], as will be discussed later.

More research is needed before these questions can be effectively answered.

Manipulating endocrinology

The role of FSH

Inherent biological mechanisms such as follicle sensitivity to FSH and pharmacodynamics of drug metabolism or receptor interaction [194] may affect the individual ovarian response to stimulation. Recent genetic and pharmacogenomic research has revealed other factors that may facilitate improved cycle management.

FSH secreted from the pituitary is a heterodimer glycoprotein hormone with two covalently linked subunits, α and β . The molecule is glycosylated by post-translational modification, and the presence and composition of the carbohydrate glycan moieties determine its *in vivo* biological activity (Figure 52.6) [195, 196]. *In vivo*, the native FSH consists of a family of up to 20 different isohormones that differ in their pattern of glycosylation. For follitropin- α , isoelectric focusing has identified seven major bands of FSH isoforms between pI 4.2 and 5.05, five minor bands



FIGURE 52.6 Follicle-stimulating hormone is a complex glycoprotein with two non-covalently associated α - and β -protein subunits. Two oligosaccharides are linked to each protein subunit. (Molecular model created by Merck Serono Reproductive Biology Unit, USA; reproduced with permission.)

between pI 5.25 and 6.30, and one minor band at pI 4.20. These have been demonstrated to be consistent between different manufactured batches [197]. The ovarian response to stimulation by FSH relies on an interaction of the hormone with membrane receptors (FSHR) on GCs, and a normal response is dependent on the correct molecular structure of the hormone, the receptor, and factors associated with their interaction. Any defect in the genes encoding FSH or its receptor may result in ovarian resistance, and

therefore genotype may play a fundamental role in determining the physiological response to FSH stimulation.

The FSHR is a member of the family of G-protein receptors linked to adenyl cyclase signalling, with extensive extracellular ligand-binding domains. The gene encoding the FSHR is located on the short arm of chromosome 2 and is made up of 2085 nucleotides that translate into a polypeptide with 695 amino acids. This molecule has four potential N-linked glycosylation sites located at amino acids 191, 199, 293, and 286. Mutations in the receptor gene can result in amino acid changes that affect function, and mutations that result in complete FSH resistance [198] as well as partial loss of FSHR function have been identified [199]. Screening different populations for mutations of the FSHR gene have shown that single nucleotide polymorphisms can be identified, and two discrete polymorphisms have been studied: (1) position 307 (Ala or Thr) in the extracellular domain; and (2) position 680 (Asn or Ser) in the intracellular domain. Both polymorphic sites give rise to two discrete allelic variants of the FSHR (i.e. Thr307/Asn680 and Ala307/Ser680). There is an association between these polymorphisms and ovarian response in patients undergoing ART [200, 201], and their frequency may vary among different ethnic groups. Women with the Ser/Ser polymorphism at position 680 have an increased total menstrual cycle length and time from luteolysis to ovulation compared with Asn/Asn controls [202]. This Ser/Ser genotype occurs less frequently in Asian women than in Caucasians (Table 52.2).

In a Korean IVF patient population, Jun et al. [201] grouped 263 young patients according to their FSHR genotype and found that basal FSH levels differed between the groups. The Ser/Ser (p.N680S) homozygous group required higher total doses of gonadotropins to achieve multiple follicular development compared with the other two groups (Asn/Asn and Asn/Ser at position 680). Additionally, significantly fewer oocytes were recovered in patients with the Ser/Ser FSHR genotype.

Perez Mayorga et al. [200] also suggest that the FSHR genotype plays a fundamental role in determining the physiological response to FSH stimulation, and that subtle differences in FSHR might fine-tune the action of FSH in the ovary. In a study conducted in 161 ovulatory young (<40 years) women who underwent IVF treatment, a wide variation in the number of ampules

TABLE 52.2 The Frequency of the Follicle-Stimulating Hormone Receptor Polymorphism at p.N680S in Published Reports

Study	Ethnic Origin	Patient Number (Diagnosis)	SNP680		
			Asn/Asn (%)	Asn/Ser (%)	Ser/Ser (%)
Perez Mayorga et al. [200]	Caucasian	161 (male/tubal)	29	45	26
Sudo et al. [205]	Japanese	522 (mixed)	41	46.9	12.1
Laven et al. [206]	Caucasian	148 (anovulatory)	16	44	40
Laven et al. [206]	Caucasian	30 (ovulatory)	23	61	16
De Castro et al. [207]	Caucasian	102 (male/tubal/both)	31.4	50	18.6
Daelemans et al. [208]	Caucasian	99 (non-IVF control)	38	45	17
Daelemans et al. [208]	Caucasian	130 (mixed?)	24	51	25
Daelemans et al. [208]	Caucasian	37 (mixed-OHSS)	16	54	32
Choi et al. [209]	Korean	172 (mixed, non-PCOS)	41.9	47.7	10.5
Schweickhardt 2004—unpublished thesis	Not stated (USA)	663 (mixed)	30.6	48.7	20.7

Note: The Ser/Ser (p.N680S) homozygous group is generally lower in Asian populations than in Caucasian populations.

Abbreviations: IVF, *in vitro* fertilization; OHSS, ovarian hyperstimulation syndrome; PCOS, polycystic ovary syndrome.

of FSH required to achieve an adequate response was observed. They confirmed that this observation could be correlated with the patient's FSHR genotype (i.e. type of polymorphism).

Behre et al. [203] also carried out an RCT to further investigate this observation and found that the Ser/Ser (p.N680S) homozygous group results in lower E2 levels following FSH stimulation. This lower FSHR sensitivity could be overcome by higher FSH doses in the trial patients.

Achrekar et al. have shown that the AA genotype at the -29 position in the 5'-untranslated region of the FSHR gene may be associated with the POR to COS [204]. Women with the AA genotype required a large total dose of exogenous FSH and only low numbers of pre-ovulatory follicles were produced and oocytes retrieved. In addition, E2 levels on the day of hCG administration were significantly lower in women with the AA versus GA genotypes.

Taken together, these studies demonstrate that the FSHr genotype does certainly modulate ovarian responsiveness to FSH and it can contribute to a lower response following COS. Knowing the FSHr polymorphism prior to stimulation start in previous POR may be helpful leading to the prospective administration of higher FSH starting doses and thus reduce the chance of there being an iatrogenic hypo response. However, a recent study has clearly demonstrated that there is no evidence that oocyte quality and pregnancy outcome is impacted by FSHr and LHr polymorphisms [210]. The variables that are consistently associated with pregnancy and live birth are the woman's age and number of oocytes retrieved.

In recent years, there has been a paradigm shift in the use of gonadotropins. The outdated "one-size-fits all" approach to fertility treatment has been superseded by individualized COS [55, 211, 212]. Individualized COS is designed to maximize the efficacy and safety for each patient and is discussed more fully in Chapter 38.

Accordingly, an analysis was undertaken to assess whether specific factors could optimally predict a response to stimulation in ART, and then to develop a corresponding treatment algorithm that could be used to calculate the optimal starting dose of r-FSH (follitropin- α) for selected patients [54]. Backwards stepwise regression modelling indicated that in ART patients aged <35 years (n = 1378) who were treated with r-FSH monotherapy,

predictive factors for ovarian response included basal FSH, BMI, age, and number of follicles <11 mm at baseline screening. The concordance probability index was 59.5% for this model. Using these four predictive factors, a follitropin- α starting dose calculator was developed that can be used to select the FSH starting dose required for an optimal response. A prospective cohort study in young normo-responding patients has been completed using this r-FSH starting dose calculator and demonstrated a similar number of oocytes and pregnancy rates across the doses used [213]. It must be emphasized that this study was carried out in young, normo-responding patients. There are no studies available in POR patients.

The role of LH

Ovulation induction studies in hypogonadotropic women using r-FSH have demonstrated that FSH can induce follicular growth to the pre-ovulatory stage, but E2 and androstenedione concentrations remain extremely low [214, 215]. This suggests that final follicular maturation depends on the action of LH to stimulate androstenedione biosynthesis as a substrate for aromatase activity. Below a minimal level of LH, follicular development may plateau or lead to a lengthening of follicular stimulation—this has been observed in patients with profound pituitary downregulation after GnRHa depot [216]. In women with hypogonadotropic hypogonadism, E2 concentrations may be inadequate for cytoplasmic maturation of the follicle, endometrial proliferation, and corpus luteum function [214, 215].

Adequate folliculogenesis and steroidogenesis required for successful fertilization and implantation therefore depend upon a certain threshold level of LH. Although the amount of LH necessary for normal follicle and oocyte development is unclear, it is likely to be very low, since a maximal steroidogenic response can be elicited when <1% of follicular LH receptors are occupied [217]. On this basis, resting levels of LH (1–10 IU/L) should be sufficient to provide maximal stimulation of thecal cells [218]. There is also evidence that excessive levels of LH can have an adverse effect on follicular development [219] associated with impaired fertilization and pregnancy rates, as well as higher miscarriage rates, through the so-called "ceiling" effect (Figure 52.7). LH

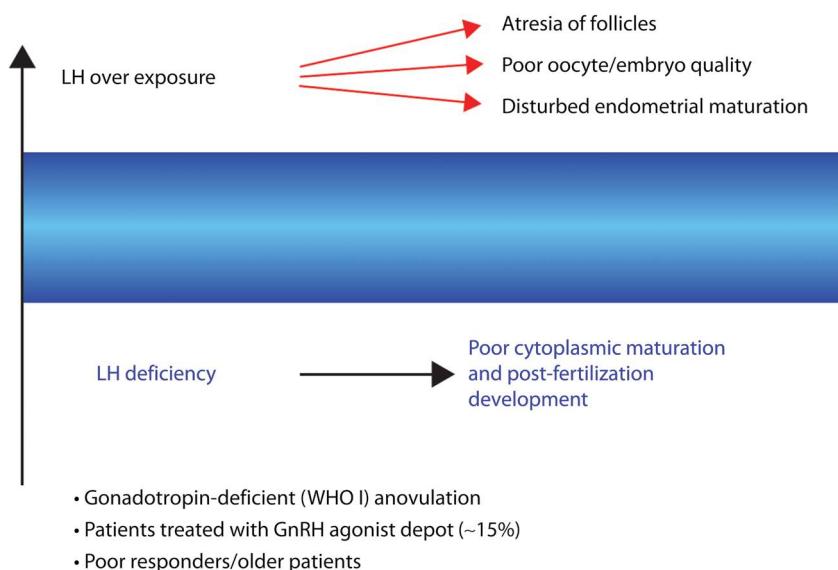


FIGURE 52.7 The LH therapeutic window concept. Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; WHO, World Health Organization.

levels must be below this ceiling in order for the LH-dependent phase of development to proceed normally. It seems that there is a clinical therapeutic window [220, 221]: “low-dose” treatment with LH generally enhances steroidogenesis, but “high-dose” treatment can enhance progesterone synthesis, suppress aromatase activity, and inhibit cell growth.

Huirne et al. [222] administered different GnRH-ant doses to five groups of patients and measured the subsequent change in LH levels between the groups. The aim of this study was to deliberately induce different LH levels and to assess the effect of an LH range on IVF outcome in order to estimate what the optimal level might be. No pregnancies were observed in relation to either very high or very low LH, suggesting an optimal window. However, their data led them to conclude that not the absolute level, but instead excessive change in LH—either increases or decreases—was the more significant parameter. They suggest that the correct sequence of stages in oocyte maturation, together with synchrony between nuclear and cytoplasmic maturation, is dependent upon an appropriate endocrine milieu. Excessive fluctuations in LH levels might disrupt this balance, as well as affect maturation of the endometrium (i.e. stable and appropriate LH levels are needed during IVF cycles). It is possible that specific patient groups, such as those with PCOS or DOR, may be prone to larger changes in LH levels and sensitive to high fluctuations. In addition, serum LH levels assayed by immunoassay do not necessarily reflect circulating LH bioactivity, particularly in these specific patient groups.

A common variant of the LH gene is recognized (Trp⁸Arg and Ile¹⁵Thr of the β-subunit) that encodes a protein with altered *in vitro* and *in vivo* activity [223]. It has been suggested that this variant may be less effective at supporting FSH-stimulated multifollicular growth, resulting in suboptimal ovarian response to standard COS regimens and higher drug consumption [224]. An increased prevalence of this gene has been reported in Japanese patients with infertility [225] and premature ovarian failure [226], and it has been postulated that women with this gene variant could benefit from exogenous LH supplementation during COS. However no clinical data is currently available to verify this hypothesis.

The initial availability of a pure r-LH (Luveris®, Merck Darmstadt, Germany) preparation has provided a new tool that allows the endocrinology of ovarian stimulation to be examined more accurately. Luveris in combination with FSH is indicated for follicular stimulation in women with severe gonadotropin deficiency. The indication was based upon a dose finding study by the European Recombinant Human LH Study Group [227]. This study demonstrated that increasing exposure to LH during the follicular phase reduces the number of growing follicles (“ceiling effect”) [219]. Additional studies further supported that 75 IU r-LH/day in combination with rFSH was sufficient for promoting optimal follicular development in the majority of HH patients [228]. Eventually, a combination follitropin alfa/lutropin alfa (150IU FSH:75IU LH, Pergoveris, Merck Darmstadt, Germany) was launched in EU in 2007 for this indication.

The clinical utility of the LH ceiling effect was further explored in a series of studies. Subsequent findings from a pilot study demonstrated that high doses of r-LH in the late follicular phase suppressed follicular development both in HH as well as WHO II anovulatory women [229].

Hugues et al. [230] investigated if r-LH could be used to achieve mono ovulation for conception *in vivo*. In this elegant placebo-controlled, double-blind study, four doses of r-LH (150, 300, 660,

1325 IU) were given daily in the late follicular phase (in combination with a fixed dose of 37.5 IU FSH) to find the optimal dose that could maintain growth of a dominant follicle, whilst leading to atresia of secondary ones. The study was conducted in WHO II anovulatory women who were experiencing an excessive ovarian response to FSH treatment. The results demonstrated that doses up to 660 IU r-LH/day increased the proportion of patients developing a single dominant follicle compared to placebo.

The use of r-LH in COS protocols for ART has been reviewed [231] extensively, and to date there is still no definitive evidence, from randomized clinical trials, that LH supplementation is beneficial in terms of ongoing pregnancy rates. Recently, there has been presented a comprehensive systematic review and network analysis on ovarian stimulation regimens [231]. Here as far as LH supplementation was concerned in ART, there was a reduction in the number of oocytes retrieved, thus clearly in line with the early studies in hypogonadotropic and PCO women. A clear relationship between the dose of r-LH and serum E2 has been found in hypogonadotropic patients [227]. The optimal LH levels required to provide the best results in IVF are still a matter of debate, and a number of studies have tried to assess the role of LH supplementation in GnRHa and GnRH-ant cycles. Considering the move from the widespread use of GnRHa protocols, where severe gonadotropin deficiency may well occur, to a shorter GnRH antagonist protocol with exposure for a period of just four to six days, the role and value of LH supplementation needs to be urgently addressed by the research community.

LH supplementation may have an effect via intraovarian mechanisms that affect steroid biosynthesis, and therefore oocyte maturation. Foong et al. [232] conducted a study that included patients who showed an inadequate response to r-FSH-only stimulation, and reported that although peak E2 levels were similar to those found in normal responders, intrafollicular E2 levels were significantly lower, and progesterone was significantly higher in poor responders to FSH. E2 plays an important role in human oocyte cytoplasmic maturation *in vitro* [233], as manifested by improved fertilization and cleavage rates. hGh has also been shown to stimulate E2 production by follicular cells [234, 235]. High intrafollicular E2 concentrations in the pre-ovulatory follicle predict an increased chance of pregnancy [236]. On the other hand, androstenedione can irreversibly block the effect of E2 [237], and it is clear that maintaining an appropriate steroid balance within the follicle is very important. In the ovine, E2 is associated with an upregulation of oocyte DNA repair enzymes [238]. In the rhesus monkey, adding an aromatase inhibitor during the late stages of follicular development, just prior to the period of ovulation, resulted in a reduced capacity of the oocyte to mature and a reduced rate of fertilization *in vitro* [239]. Overall, it seems that LH may have a beneficial effect through a mechanism that improves oocyte cytoplasmic maturation (increasing mitochondrial function and/or upregulating DNA repair enzymes), either through E2 or some other intraovarian factor. However, an additional effect on the endometrium itself cannot be excluded.

A number of further studies have examined the effect of LH supplementation in poor responders [240] or patients who respond inadequately to FSH stimulation [216, 241, 242]. Following stratification of the data, a subset of patients aged ≥35 years were identified who seem to benefit from LH supplementation in terms of an increased number of mature oocytes retrieved and improved implantation and pregnancy rates. This benefit was maintained even when LH supplementation was initiated from stimulation days 6 or 8. This seems logical in terms of physiology, as the

GCs, through FSH stimulation, acquire LH receptors only after the follicle reaches a diameter of at least 11 mm [243]. However in the ESPART trial [244], the largest RCT study carried out in POR patients defined according to the Bologna criteria, no benefit in terms of number of oocytes retrieved or ongoing clinical pregnancy rates were found when LH supplementation (using a FSH:LH, 2:1 ratio product) was compared to FSH alone. Whilst it is clear now that the Bologna criteria encompass a heterogeneous patient population and that applying the POSEIDON criteria may well define better those who will benefit from LH supplementation, a recently published real-world evidence study on over 9000 low-prognosis patients classified according to the POSEIDON criteria [245] did not demonstrate a benefit of LH supplementation on outcomes. A logistic regression analysis revealed that the POSEIDON grouping, number of embryos obtained, number of ET cycles per patient, number of oocytes collected, female age, duration of infertility, and BMI were relevant predictors for cumulative delivery rate CDR ($P < 0.001$). Gonadotropin type, total gonadotropin dose, type of GnRH analogue, ovulation trigger were not significantly associated with CDR.

In hypo-responsive women, the need for higher FSH doses might be an individual biological index of LH deficiency, with an effect on oocyte competence, however this hypothesis requires clinical trial validation.

A requirement for LH supplementation in order to achieve good ovarian response and follicular maturation in patients of AMA could be based on a number of theoretical explanations. With age and the onset of the menopause, endogenous LH as well as FSH levels increase and T levels decrease [246, 247]. The number of functional LH receptors also decreases with age [248]. Kim et al. [249] found that the best predictor of ovarian reserve (reproductive age) in normally cycling women was the combination of the FSH and LH levels on menstrual cycle day 1. There is also evidence that endogenous LH may be less biologically active or potent than it should be, or the immunologic LH may not be comparable to the biologically active LH [250, 251]. Overall, this could result in increasing ovarian resistance to LH-mediated events.

It has been suggested that follicular recruitment in women aged >38 years can be improved by supplementing r-FSH stimulation with LH-containing preparations [252, 253]. Since hMG contains hCG and a number of unknown contaminating proteins in addition to FSH and LH, Gomez-Palomares et al. [254] conducted a prospective randomized cohort study comparing the effects of hMG with r-LH supplementation in a group of women aged 38–40 years in order to determine whether LH is the hMG component that favours early follicular recruitment. The patients were randomly assigned to one of two groups: 58 patients received r-FSH 225 plus hMG (one ampule), and 36 were treated with r-FSH 225 plus r-LH 75 until day 6. Follicular recruitment was evaluated on day 6, and stimulation was continued with r-FSH alone, without further hMG or r-LH. Both groups recruited a similar number of follicles after five days of stimulation, but the r-LH group showed a significant increase in the number of metaphase II oocytes retrieved and a higher clinical pregnancy rate (47% vs 26%; non-significant). Another meta-analysis [255] reported that although in women ≥ 35 years of age, fewer oocytes were retrieved with r-FSH/r-LH versus r-FSH monotherapy, the clinical pregnancy rate was reported to be higher in the r-LH supplemented groups. An RCT in patients stimulated with different FSH starting doses, but similar total IU of FSH+LH activity, demonstrated no differences in ongoing clinical pregnancy rates in women aged 36–39 years and in those <36 years [256]. Other

studies failed to find any difference with LH supplementation [257, 258].

In a group of patients representing about 10%–15% of young women, ovarian response to COS using r-FSH in GnRHa protocols is suboptimal (rather than poor), despite the presence of normal circulating FSH and/or LH levels [259]. Such patients have normal follicular development up to cycle days 5–7, but this response plateaus on days 8–10. As described previously, suboptimal ovarian response to FSH may be due to an LH- β variant polymorphism [260], or to polymorphic variants of the FSH receptor [194, 261]. Early evidence suggests that LH supplementation may improve outcomes in patients with suboptimal response to FSH stimulation [216, 240, 242]. Lisi et al. showed a significant improvement in fertilization and clinical pregnancy rates with the addition of r-LH to r-FSH in women who required high doses of r-FSH in previous cycles [240]. Ferraretti et al. demonstrated that supplementation from the mid-to-late stimulation phase with r-LH but not hMG was associated with significantly improved implantation and clinical pregnancy rates in patients who responded inadequately to FSH-only stimulation [242]. This is an interesting category of ART patients and it seems that such a response may be more common in a GnRHa depot regimen [216, 241]. De Placido and colleagues also described the beneficial use of r-LH supplementation administered following the occurrence of a plateau in E2 secretion and a lack of continued follicle growth at around day 7 of FSH stimulation [216, 241].

Several studies have also supported the need for additional LH in poor responders when short and long protocols of GnRHa are used [263–265]. From such studies, it has been theorized that ovarian stimulation in patients with diminished ovarian reserve may be enhanced by the LH-induced production of E2 precursors such as androstenedione.

Because of the sudden and often dramatic inhibition of LH secretion associated with the use of GnRH-ant, there has been interest in the potential need for exogenous LH supplementation. A recent meta-analysis of data on 1764 women (aged 18–39 years) from six RCTs showed that the amount of endogenous LH during GnRH-ant protocols was sufficient to support r-FSH in COS prior to IVF or ICSI [266]. No association between endogenous LH level and pregnancy rate in normogonadotropic women was found [266].

There is, however, a paucity of data on the potential use of LH supplementation in poor responders or patients with AMA undergoing COS using a GnRH-ant protocol. In a retrospective cohort study [267], 240 GnRH-ant cycles in poor responders were evaluated. Of 153 that reached the stage of oocyte retrieval, 75 patients received r-FSH for ovarian stimulation, and 66 received hMG in combination with r-FSH. In patients aged <40 years, there were no significant differences between treatment groups in the amount and duration of treatment, number of oocytes retrieved, and number of embryos. In patients aged ≥ 40 years, significantly fewer oocytes were retrieved in patients who received exogenous LH in their stimulation, resulting in significantly fewer fertilized embryos. Implantation and clinical pregnancy rates did not differ by treatment group. It was concluded that outcomes in poor responders undergoing IVF with GnRH-ants are comparable whether COS is performed with or without supplementary LH. Berker et al. retrospectively evaluated the clinical utility of providing LH-activity supplementation to r-FSH stimulation in a cohort of 558 women, consisting mainly of POSEIDON groups 3 and 4 [268]. In their study, the addition of hMG to r-FSH from the early follicular phase was associated with higher live birth rates

(21.9% vs 11.6%, $p = 0.03$) per initiated cycle than recombinant FSH alone or hMG added from the mid-follicular phase onwards.

Similar results from an RCT using a GnRHa flare-up protocol were reported by Barrenetxea et al. [269]. Patients ($n = 84$) who had a basal FSH level of >10 mIU/mL, were aged >40 years, and undergoing their first IVF cycle were randomly allocated into two study groups: group A, in which ovarian stimulation included GnRHa flare-up and r-FSH and r-LH; and group B, in which patients received no LH. The overall pregnancy rate was 22.6%. The pregnancy wastage rate was 30.0% in group A and 22.2% in group B. There were no differences in the ongoing pregnancy rate per retrieval and implantation rate per ET. The duration of stimulation, E2 level on hCG administration day, number of developed follicles, number of retrieved oocytes, number of normally fertilized zygotes, cumulative embryo score, and number of transferred embryos were all comparable for the two groups. It was concluded that the addition of r-LH at a given time of follicular development produces no further benefit in poor-responder patients stimulated with the short protocol, and a reduced ovarian response cannot be overcome by changes in the COS protocol.

A Cochrane systematic review reported that a statistical difference was not found in clinical or ongoing pregnancy rates in all ART patients who received r-FSH alone or r-FSH plus r-LH [193]. However, a sub-analysis of data on poor responders from three trials using GnRHa protocols showed a significant increase in the ongoing pregnancy rate in favour of co-administration of r-LH (odds ratio [OR] 1.85, 95% CI 1.10–3.11). The authors recommended further work to elucidate a potentially beneficial effect of r-LH in poor responders. A recent update of the aforementioned meta-analysis concluded that since the sample size for the subgroup analysis in women with POR and in women of advanced age was small, there was insufficient evidence to make a conclusive judgement of any beneficial effect of r-LH combined with r-FSH in IVF or ICSI cycles compared to rFSH alone in these women [270].

To summarize all of the different findings over the years, as of 2023, it is still not clear what patient group may benefit from LH supplementation. As stated earlier, the value of LH supplementation needs to be urgently addressed by the research community.

OCP pre-treatment

It has been suggested that the use of an OCP in the previous cycle may increase pregnancy rates in IVF [271]. Because OCPs have a putative role in the enhancement of oestrogen receptor sensitization due to their oestrogen content, in addition to exerting pituitary suppression, they have been used in combination with GnRHa. Biljan et al. [272] reported that pituitary suppression with OCP and a GnRHa was superior to GnRHa alone regarding the time required to achieve pituitary suppression, as well as pregnancy and implantation rates.

Because of these promising effects, OCPs have also been used in poor responders. However, there are only very few retrospective studies evaluating the actual contribution of OCPs in this group of patients. Lindheim et al. [273] found higher pregnancy rates with OCP alone compared with GnRHa-treated cycles (both long and short protocols). They concluded that the good outcome associated with OCP pre-treatment might reflect the production or alteration of local ovarian growth factors and/or changes at the endometrial level. In contrast with the preceding observations, Kovacs et al. [274] also retrospectively compared the use of OCPs with GnRHa for hypothalamic–pituitary suppression in poor responder IVF patients. Hypothalamic–pituitary suppression was

performed with either an OCP or a GnRHa followed by stimulation with gonadotropins. Cycle outcomes, including cancellation rates, gonadotropin requirements, number of oocytes retrieved, number of embryos transferred, and embryo quality, were similar. Patients in the OCP group required fewer days of stimulation to reach oocyte retrieval. Pregnancy rates were similar in the two groups. Overall, there was no improvement in IVF cycle outcome in poor responders who received OCPs to achieve pituitary suppression instead of a GnRHa.

In summary, although there is a general feeling that OCP pre-treatment might be of assistance in the ovarian response of poor responders, especially in flare-up regimens, only a minimal amount of published data exists to support this approach.

Luteal-phase manipulations

During the early follicular phase of the menstrual cycle, antral follicle sizes are often markedly heterogeneous. These follicle size discrepancies may, at least in part, result from the early exposure of FSH-sensitive follicles to gradient FSH concentrations during the preceding luteal phase. This phenomenon, which often occurs in women with poor ovarian reserve, and in particular those with short cycles, may potentially affect the results of ovarian stimulation. Pre-existing follicle size discrepancies may encumber coordinated follicular growth during ovarian stimulation, thereby reducing the number of follicles that reach maturation at once. Interventions aimed at coordinating follicular growth by manipulation at the mid-luteal phase of the preceding cycle are largely based on the innovative work of Fanchin et al. [275].

To investigate this issue, three clinical studies were conducted to test the hypothesis that luteal FSH suppression could coordinate subsequent follicular growth. First, luteal FSH concentrations were artificially lowered by administering physiological E2 doses and follicular characteristics were measured on the subsequent day 3 in healthy volunteers [276]. In this study, luteal E2 administration was found to reduce the size and to improve the homogeneity of early antral follicles on day 3.

Subsequently, it was verified whether luteal E2 administration could promote the coordination of follicular growth during ovarian stimulation and improve its results [277]. Ninety IVF patients were randomly pre-treated with 17 β -oestradiol (4 mg/day) from cycle day 20 until next cycle day 2 ($n = 47$) or controls ($n = 43$). On cycle day 3, all women started r-FSH treatment followed by a GnRH-ant in the flexible protocol. The authors focused on the dynamics of follicular development, including magnitude of size discrepancy of growing follicles on day 8 of r-FSH treatment and number of follicles >16 mm in diameter on the day of hCG administration. On day 8, follicles were significantly smaller (9.9 ± 2.5 vs 10.9 ± 3.4 mm) and their size discrepancies were attenuated in the treatment group compared with controls. This was associated with more >16 -mm follicles and more mature oocytes and embryos in the E2-treated group. It was concluded that luteal E2 administration reduces the pace of growth, improves size homogeneity of antral follicles on day 8 of r-FSH treatment, and increases the number of follicles reaching maturation at once. A recently published meta-analysis on the role of luteal E2 priming in poor responders found a significantly lower cycle cancellation rate among women with luteal E2 priming. This meta-analysis comprised pooled results of eight studies, of which only one study was an RCT [278].

The effects of premenstrual GnRH-ant administration on follicular characteristics were assessed during the early follicular phase [279]. Twenty-five women underwent measurements of early antral

follicles by ultrasound and serum FSH and ovarian hormones on cycle day 2 (control/day 2). On day 25, they received a single dose of 3 mg cetrorelix acetate. On the subsequent day 2 (premenstrual GnRH-ant/day 2), participants were re-evaluated as on control/day 2. The main outcome measure was the magnitude of follicular size discrepancies. Follicular diameters (4.1 ± 0.9 vs 5.5 ± 1.0 mm) and follicle-to-follicle size differences decreased on premenstrual GnRH-ant/day 2 compared with control/day 2. Consistently, FSH (4.5 ± 1.9 vs 6.7 ± 2.4 mIU/mL), E2 (23 ± 13 vs 46 ± 26 pg/mL), and inhibin-B (52 ± 30 vs 76 ± 33 pg/mL) were lower on GnRH-ant/day 2 than on control/day 2. It was concluded that premenstrual GnRH-ant administration reduces diameters and size disparities of early antral follicles, probably through the prevention of luteal FSH elevation and early follicular development.

Taken together, the results of the preceding studies suggest that luteal FSH suppression by either E2 or GnRH-ant administration could improve the size homogeneity of early antral follicles during the early follicular phase, an effect that persists during ovarian stimulation. Coordination of follicular development could have the potential to optimize ovarian response to COS protocols, and constitutes an attractive approach for improving their outcome, which needs to be evaluated in well-designed RCTs.

An opposite approach of enhancing follicular recruitment by initiating FSH therapy during the late luteal as opposed to the early follicular phase has been attempted in prior poor responders but without success. In an RCT, Rombauts et al. [280] failed to demonstrate any benefit of this regimen, with the exception that follicular maturation was achieved sooner after the onset of menses.

Several studies evaluated the effects of combining pre-treatment with E2 and/or GnRH-ant during the luteal phase of the preceding cycle on the outcome of COS in poor responders. Dragistic et al. [281] reported lower cancellation rates and improved IVF outcomes via a combination of oestrogen patch therapy and GnRH-ant started in the mid-luteal phase of the preceding menstrual cycle. Frattarelli et al. [282] reported a retrospective paired cohort analysis where they compared embryo and oocyte data between a standard protocol and a luteal-phase E2 protocol. The results of 60 poor-responder patients who underwent IVF with a luteal-phase oral E2 protocol were compared to 60 cycles in the same patients without E2 pre-treatment. The luteal-phase E2 protocol showed significant increases in the number of embryos with more than seven cells, number of oocytes retrieved, number of mature oocytes, and number of embryos generated than did the standard protocol. There was no difference between the two protocols with respect to basal AFC, days of stimulation, number of follicles >14 mm on day of hCG administration, or endometrial thickness. A trend towards improved pregnancy outcomes was found with the luteal-phase E2 protocol.

Several studies compared the luteal-phase E2 protocol with a subsequent GnRH-ant protocol with the short microflare agonist protocol. DiLuigi et al. [283] performed an RCT to compare IVF outcomes in 54 poor-responder patients undergoing a microdose LA flare protocol or a GnRH-ant protocol incorporating both a luteal-phase E2 patch and GnRH-ant in the preceding menstrual cycle. Cancellation rates (32.1% vs 23.1%), number of oocytes retrieved (5.4 ± 4.7 vs 5.2 ± 4), clinical pregnancy rates (28.6% vs 34.6%), and ongoing pregnancy rates (25% vs 23.1%) were similar for the microflare and luteal E2/GnRH-ant protocols, respectively. Similarly, Weitzman et al. [284] retrospectively compared IVF outcomes in poor responder patients undergoing COS after luteal-phase E2 patch and subsequent GnRH-ant protocol ($n = 45$)

versus microdose GnRH-ant flare protocol ($n = 76$). The cancellation rate (28.9% vs 30.3%), mean number of oocytes (9.1 ± 4.1 vs 8.9 ± 4.3), fertilization rate ($70.0 \pm 24.2\%$ vs $69.9 \pm 21.5\%$), number of embryos transferred (2.5 ± 1.1 vs 2.7 ± 1.3), implantation rate (15.0% vs 12.5%), clinical pregnancy rate (43.3% vs 45.1%), and ongoing pregnancy rate per transfer (33.3% vs 26.0%) were all comparable for both groups. Focusing on young poor responders (aged <35 years), Shastri et al. [285] retrospectively compared COS with a luteal E2 and subsequent GnRH-ant protocol versus an OCP microdose LA flare protocol. Patients in the luteal E2/GnRH-ant group had increased gonadotropin requirements (71.9 ± 22.2 vs 57.6 ± 25.7 ampoules) and lower E2 levels (1178.6 ± 668 vs 1627 ± 889 pg/mL), yet achieved similar numbers of oocytes retrieved and fertilized, and a greater number of embryos transferred (2.3 ± 0.9 vs 2.0 ± 1.1), with a better mean grade (2.14 ± 0.06 vs 2.70 ± 1.80) compared with the microflare group. The luteal E2/GnRH-ant group exhibited a trend toward improved implantation rates (30.5% vs 21.1%) and ongoing pregnancy rates per started cycle (37% vs 25%). From the above studies [283–285], it can be concluded that both protocols remain viable options for poor responders undergoing IVF, and that adequately powered, randomized clinical comparison appears justified.

When luteal E2 and antagonist ($n = 256$) was compared with luteal E2 only ($n = 57$) before a GnRH-ant protocol in low responders [286], the addition of GnRH-ant to luteal E2 for luteal suppression did not improve IVF outcome.

Elassar et al. [287] compared IVF outcomes after COS using letrozole/antagonist (LA) versus luteal phase E2/GnRH-ant in poor responders. In a retrospective study, 99 women with two or more prior failed cycles with poor response were included. In the luteal intervention group ($n = 52$), both transdermal E2 and GnRH-ant were administered in the preceding luteal phase, with gonadotropins started on the second day of menstruation. In the LA group ($n = 47$), letrozole 5 mg/day was initiated on the second day of spontaneous menstruation for five days, then gonadotropins were added on day 5; for both groups, a flexible antagonist protocol was used. The total dose of gonadotropins administered and E2 levels on the day of hCG administration were significantly lower with the LA protocol. Cancellation rate (55.3% vs 36.5%), number of oocytes retrieved (6.1 ± 3.0 vs 7.9 ± 4.8), number of transferred embryos (2.2 ± 1.0 vs 2.4 ± 1.4), and ongoing pregnancy rate per transfer (40% vs 21.2%) and per initiated cycle (19.1% vs 13.5%) were similar in the LA and luteal intervention groups, respectively. It was concluded that both aromatase inhibitor regimens and luteal intervention regimens can be feasible alternatives in recurrent POR.

Using a slightly different approach, Fisch et al. [164] described their experience with a protocol using AACEP in poor responders with prior IVF failures. The AACEP protocol focuses on promoting estrogenic dominance in the stimulated ovary and opposing the potential ill effects of the LH flare and overproduction of androgens, which are commonly seen in GnRH-ant flare and in antagonist protocols. Patients received an OCP and a GnRH-ant overlapping the last five to seven days of the pill until the onset of menses. From cycle day 2, low-dose GnRH-ant (0.125 mg/day) and oestradiol valerate (2 mg) were given intramuscularly every three days for two doses, followed by oestrogen suppositories until a dominant follicle was detected. Ovarian stimulation consisted of high-dose FSH/hMG. Although women aged <38 years and those on 600 IU/day produced more mature eggs and fertilized embryos than women aged 38–42 years, there were no differences in peak serum E2, endometrial thickness, or embryos

transferred. Outcomes were similar for all patients, regardless of age or FSH dosage. Ongoing pregnancy rates were 27% for all patients, 25% for patients aged <38 years, and 28% for patients aged 38–42 years. It was concluded that the AACEP protocol may improve the prognosis and outcomes for poor responders with prior IVF failures.

In summary, manipulating the luteal phase preceding the IVF treatment cycle may improve the coordination of follicular development and increase the number and quality of embryos achieved in poor-responder patients. Ultimately, this may translate into improved cycle and pregnancy outcomes in these patients. It remains to be seen whether this approach is superior to pre-treatment with an OCP, which is commonly practiced in various protocols designed for poor responders. Properly designed RCTs are needed to test this innovative therapeutic approach.

Recent strategies

Oocyte accumulation and embryo banking

Improvements in cryopreservation and vitrification techniques have led to the increased uptake of elective oocyte and embryo cryopreservation with deferred ET with the advantage of avoiding the risk of OHSS without jeopardizing pregnancy outcomes [286, 287]. In a prospective study, Cobo et al. [288] have demonstrated that the strategy of oocyte accumulation could increase the inseminated cohort in poor responders, thereby creating a similar situation to normal responders. The study included 242 low-responder (LR) patients (594 cycles) whose mature oocytes were accumulated by vitrification and inseminated simultaneously (LR-Accu-Vit) and 482 patients (588 cycles) undergoing IVF-ET with fresh oocytes in each stimulation cycle (LR-fresh). The dropout rate in the LR-fresh group was >75%. The ET cancellation rate per patient was significantly lower in the LR-Accu-Vit group (9.1%) than the LR-fresh group (34.0%). The live birth rate/patient was higher in the LR-Accu-Vit group (30.2%) than the LR-fresh group (22.4%). The cumulative live birth rate/patient was statistically higher in the LR-Accu-Vit group (36.4%) than the LR-fresh group (23.7%), and a similar outcome was observed among patients aged ≥40 years (LR-Accu-Vit 15.8% vs LR-fresh 7.1%). The LR-Accu-Vit group had more cycles with embryo cryopreservation (LR-Accu-Vit 28.9% vs LR-fresh 8.7%). The authors' conclusion was that accumulation of oocytes by vitrification and simultaneous insemination represents a successful alternative for LR patients, yielding comparable success rates to those in normal responders and avoiding adverse effects of a low response. Further studies supporting oocyte and embryo accumulations have been recently published [289, 290].

Summary: Practical considerations

There are several key issues that make the development of treatment strategies for poor-responder patients difficult and frustrating:

1. Historically, there was no universally accepted definition of POR until the ESHRE definition [12] and now the POSEIDON grouping. Although many papers referenced in this text use a large variety of inclusion criteria and are therefore not readily comparable, it is hoped that future studies using standardized definitions will provide more reliable evidence.

2. There is still a need for large-scale RCTs (ideally double blinded) to test the efficacy of interventions such as LH, T, and hGH supplementation.

The following practical considerations represent a combination of the evidence presented earlier with long-standing clinical experience.

High-dose gonadotropins

Patients with either diminished ovarian reserve (by testing prior to treatment) or POR in previous cycles may benefit from high-dose gonadotropin therapy (300 FSH daily) in order to maximize oocyte yield.

Long GnRHa protocol

The long GnRHa protocol is one of the protocols that can certainly be offered to poor responders. However, the increased treatment burden and gonadotropin requirements (LH supplementation or not) have to be carefully balanced. If the long protocol is to be used, progestogen pre-treatment may reduce the incidence of cyst formation. Reducing the dose of the GnRHa once pituitary down-regulation has been achieved (mini-dose agonist) is one suggested strategy with the long GnRHa regimen.

GnRH-ant protocol

The GnRH-ant protocol is nowadays considered the protocol of choice for poor responders. The GnRH-ant protocol results in lower gonadotropin consumption and shorter duration of stimulation compared to the long GnRHa protocol.

Short or microdose flare GnRHa protocol

The short GnRHa protocol can also be applied in COS regimens for poor responders. Oral contraceptive pre-treatment is an important consideration with the use of short GnRHa regimens, as it may prevent the adverse effects of elevated LH and androgen secretion caused by the endogenous gonadotropin flare. Reducing the dose of the GnRHa to microdoses, as is done in microflare regimens, is an effective and popular approach in stimulating poor responders.

Conclusions

Women who have entered the declining years of fecundity and then require assisted reproduction have always been a major challenge in ART treatment. The poor response that is commonly observed in women of AMA is directly related to diminished ovarian reserve. The associated reduction in oocyte quality as manifested by the increase in aneuploid embryos is most likely due to suboptimal cytoplasmic maturation (including reduced capacity of oocyte mitochondria to generate sufficient quantities of energy required for fertilization and cell division). In addition to the obstacles of diminished ovarian reserve, resistance to ovarian stimulation, and higher frequency of potential gynaecological disorders, these women are also at higher risk of producing aneuploid oocytes and embryos. Uterine factors, along with the possibility of aneuploid embryos, result in an increased miscarriage rate. Their situation is further compounded by the psychological stress of knowing that the "biological clock" is ticking, and that time is against them.

Although the use of donor oocytes has proved to be a very successful alternative treatment, this is not an option in many parts of the world, and efforts must be made to maximize each patient's potential to use her own oocytes. If a sufficient number

of oocytes and embryos can be obtained, aneuploidy screening by pre-implantation genetic testing could be considered. However, the role of pre-implantation genetic screening for aneuploidy (PGT-A) needs to be evaluated in poor responders following the introduction of newer and more reliable techniques for genetic testing. In the future, accurate non-invasive methods for assessing oocyte and embryo quality may also become available, such as gene expression profiling of the cumulus cells surrounding the oocyte, along with metabolomics and proteomics. These strategies, utilizing pharmacogenomics and manipulating endocrinology, may provide a means of augmenting follicular recruitment and cytoplasmic integrity, and thus improve the prognosis for these women.

References

- Wallace WH, Kelsey TW. Ovarian reserve and reproductive age may be determined from measurement of ovarian volume by transvaginal sonography. *Hum Reprod.* 2004;19(7):1612–7.
- Borini A, Bafaro G, Violini F, et al. Pregnancies in postmenopausal women over 50 years old in an oocyte donation program. *Fertil Steril.* 1995;63(2):258–61.
- Laufer N, Simon A, Samueloff A, Yaffe H, Milwidsky A, Gielchinsky Y. Successful spontaneous pregnancies in women older than 45 years. *Fertil Steril.* 2004;81(5):1328–32.
- Gielchinsky Y, Mazor M, Simon A, et al. Natural conception after age 45 in bedouin women, a uniquely fertile population. *J Assist Reprod Genet.* 2006;23(7-8):305–9.
- Spandorfer SD, Bendikson K, Dragisic K, et al. Outcome of in vitro fertilization in women 45 years and older who use autologous oocytes. *Fertil Steril.* 2007;87(1):74–6.
- Baker TG. Radiosensitivity of mammalian oocytes with particular reference to the human female. *Am J Obstetr Gynecol.* 1971;110(5):746–61.
- Gougeon A. Dynamics of follicular growth in the human: A model from preliminary results. *Hum Reprod.* 1986;1(2):81–7.
- De Geyter C, Calhaz-Jorge C, Kupka MS, et al. ART in europe, 2015: Results generated from European registries by ESHRE. *Hum Reprod Open.* 2020;2020(1):h0z038.
- CDC. 2019 National ART Summary. 2019. Available from: <https://www.cdc.gov/art/reports/archive.html>.
- CDC. 2013 National Summary Report. 2013. Available from: <https://www.cdc.gov/art/reports/archive.html>.
- Alvigg C, Humaidan P, Howles CM, et al. Biological versus chronological ovarian age: Implications for assisted reproductive technology. *Reprod Biol Endocrinol.* 2009;7:101.
- Surrey ES, Schoolcraft WB. Evaluating strategies for improving ovarian response of the poor responder undergoing assisted reproductive techniques. *Fertil Steril.* 2000;73(4):667–76.
- Polyzos NP, Devroey P. A systematic review of randomized trials for the treatment of poor ovarian responders: Is there any light at the end of the tunnel? *Fertil Steril.* 2011;96(5):1058–61.e7.
- Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L. ESHRE consensus on The definition of 'poor response' to ovarian stimulation for in vitro fertilization: The Bologna criteria. *Hum Reprod.* 2011;26(7):1616–24.
- Papathanasiou A. Implementing the ESHRE 'poor responder' criteria in research studies: Methodological implications. *Hum Reprod.* 2014;29(9):1835–8.
- La Marca A, Grisendi V, Giulini S, et al. Live birth rates in the different combinations of the Bologna criteria poor ovarian responders: A validation study. *J Assist Reprod Genet.* 2015;32(6):931–7.
- Busnelli A, Papaleo E, Del Prato D, et al. A retrospective evaluation of prognosis and cost-effectiveness of IVF in poor responders according to the Bologna criteria. *Hum Reprod.* 2015;30(2):315–22.
- Poseidon G, Alvigg C, Andersen CY, et al. A new more detailed stratification of low responders to ovarian stimulation: From a poor ovarian response to a low prognosis concept. *Fertil Steril.* 2016;105(6):1452–3.
- Humaidan P, Alvigg C, Fischer R, Esteves SC. The novel POSEIDON stratification of 'Low prognosis patients in Assisted Reproductive Technology' and its proposed marker of successful outcome. *F1000Res.* 2016;5:2911.
- Chuang CC, Chen CD, Chao KH, et al. Age is a better predictor of pregnancy potential than basal follicle-stimulating hormone levels in women undergoing in vitro fertilization. *Fertil Steril.* 2003;79(1):63–8.
- McClure N, McQuinn B, McDonald J, et al. Body weight, body mass index, and age: Predictors of menotropin dose and cycle outcome in polycystic ovarian syndrome? *Fertil Steril.* 1992;58(3):622–4.
- Nahum R, Shifren JL, Chang Y, et al. Antral follicle assessment as a tool for predicting outcome in IVF—is it a better predictor than age and FSH? *J Assist Reprod Genet.* 2001;18(3):151–5.
- Pohl M, Hohlagschwandner M, Obruc A, et al. Number and size of antral follicles as predictive factors in vitro fertilization and embryo transfer. *J Assist Reprod Genet.* 2000;17(6):315–8.
- Popovic-Todorovic B, Loft A, Lindhard A, et al. A prospective study of predictive factors of ovarian response in 'standard' IVF/ICSI patients treated with recombinant FSH. A suggestion for a recombinant FSH dosage nomogram. *Hum Reprod.* 2003;18(4):781–7.
- Scheffer GJ, Broekmans FJ, Loosman CW, et al. The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. *Hum Reprod.* 2003;18(4):700–6.
- Broekmans FJ, de Ziegler D, Howles CM, et al. The antral follicle count: Practical recommendations for better standardization. *Fertil Steril.* 2010;94(3):1044–51.
- Klinkert ER, Broekmans FJ, Loosman CW, et al. The antral follicle count is a better marker than basal follicle-stimulating hormone for the selection of older patients with acceptable pregnancy prospects after in vitro fertilization. *Fertil Steril.* 2005;83(3):811–4.
- Klinkert ER, Broekmans FJ, Loosman CW, et al. Expected poor responders on the basis of an antral follicle count do not benefit from a higher starting dose of gonadotrophins in IVF treatment: A randomized controlled trial. *Hum Reprod.* 2005;20(3):611–5.
- Toner JP, Philpot CB, Jones GS, Muasher SJ. Basal follicle-stimulating hormone level is a better predictor of in vitro fertilization performance than age. *Fertil Steril.* 1991;55(4):784–91.
- Abdalla H, Thum MY. An elevated basal FSH reflects a quantitative rather than qualitative decline of the ovarian reserve. *Hum Reprod.* 2004;19(4):893–8.
- Bancsi LF, Broekmans FJ, Mol BW, et al. Performance of basal follicle-stimulating hormone in the prediction of poor ovarian response and failure to become pregnant after in vitro fertilization: A meta-analysis. *Fertil Steril.* 2003;79(5):1091–100.
- Fenichel P, Grimaldi M, Olivero JF, et al. Predictive value of hormonal profiles before stimulation for in vitro fertilization. *Fertil Steril.* 1989;51(5):845–9.
- Gurgan T, Urman B, Yarali H, Duran HE. Follicle-stimulating hormone levels on cycle day 3 to predict ovarian response in women undergoing controlled ovarian hyperstimulation for in vitro fertilization using a flare-up protocol. *Fertil Steril.* 1997;68(3):483–7.
- Scott RT, Toner JP, Muasher SJ, et al. Follicle-stimulating hormone levels on cycle day 3 are predictive of in vitro fertilization outcome. *Fertil Steril.* 1989;51(4):651–4.
- Tinkanen H, Blauer M, Laippala P, et al. Prognostic factors in controlled ovarian hyperstimulation. *Fertil Steril.* 1999;72(5):932–6.
- Fawzy M, Lambert A, Harrison RF, et al. Day 5 inhibin B levels in a treatment cycle are predictive of IVF outcome. *Hum Reprod.* 2002;17(6):1535–43.
- Bancsi LF, Broekmans FJ, Eijkemans MJ, et al. Predictors of poor ovarian response in in vitro fertilization: A prospective study comparing basal markers of ovarian reserve. *Fertil Steril.* 2002;77(2):328–36.

38. Eldar-Geva T, Margalioth EJ, Ben-Chetrit A, et al. Serum inhibin B levels measured early during FSH administration for IVF May be of value in predicting the number of oocytes to be retrieved in normal and low responders. *Hum Reprod.* 2002;17(9):2331–7.
39. Ficicioglu C, Kutlu T, Demirbasoglu S, Mulayim B. The role of inhibin B as a basal determinant of ovarian reserve. *Gynecol Endocrinol.* 2003;17(4):287–93.
40. Seifer DB, Lambert-Messerlian G, Hogan JW, et al. Day 3 serum inhibin-B is predictive of assisted reproductive technologies outcome. *Fertil Steril.* 1997;67(1):110–4.
41. Nelson SM, Yates RW, Fleming R. Serum anti-mullerian hormone and FSH: Prediction of live birth and extremes of response in stimulated cycles—implications for individualization of therapy. *Hum Reprod.* 2007;22(9):2414–21.
42. Lekamge DN, Barry M, Kolo M, et al. Anti-mullerian hormone as a predictor of IVF outcome. *Reprod Biomed Online.* 2007;14(5):602–10.
43. Kwee J, Schats R, McDonnell J, et al. Evaluation of anti-mullerian hormone as a test for the prediction of ovarian reserve. *Fertil Steril.* 2007;90(3):737–43.
44. Feyereisen E, Mendez Lozano DH, et al. Anti-mullerian hormone: Clinical insights into a promising biomarker of ovarian follicular status. *Reprod Biomed Online.* 2006;12(6):695–703.
45. Nelson SM, Messow MC, Wallace AM, et al. Nomogram for the decline in serum antimullerian hormone: A population study of 9,601 infertility patients. *Fertil Steril.* 2011;95(2):736–41.e1–3.
46. van Disseldorp J, Lambalk CB, Kwee J, et al. Comparison of inter- and intra-cycle variability of anti-mullerian hormone and antral follicle counts. *Hum Reprod.* 2010;25(1):221–7.
47. Barad DH, Weghofer A, Gleicher N. Comparing anti-mullerian hormone (AMH) and follicle-stimulating hormone (FSH) as predictors of ovarian function. *Fertil Steril.* 2009;91(4 Suppl): 1553–5.
48. Broer SL, Dolleman M, van Disseldorp J, et al. Prediction of An excessive response in in vitro fertilization from patient characteristics And ovarian reserve tests And comparison in sub-groups: An individual patient data meta-Analysis. *Fertil Steril.* 2013;100(2):420–9.e7.
49. Broer SL, van Disseldorp J, Broeze KA, et al. Added value of ovarian reserve testing on patient characteristics in the prediction of ovarian response and ongoing pregnancy: An individual patient data approach. *Hum Reprod Update.* 2013;19(1):26–36.
50. Nelson SM, Yates RW, Lyall H, et al. Anti-mullerian hormone-based approach to controlled ovarian stimulation for assisted conception. *Hum Reprod.* 2009;24(4):867–75.
51. Nardo LG, Gelbaya TA, Wilkinson H, et al. Circulating basal anti-mullerian hormone levels as predictor of ovarian response in women undergoing ovarian stimulation for in vitro fertilization. *Fertil Steril.* 2009;92(5):1586–93.
52. Yates AP, Rustamov O, Roberts SA, et al. Anti-mullerian hormone-tailored stimulation protocols improve outcomes whilst reducing adverse effects and costs of IVF. *Hum Reprod.* 2011;26(9):2353–62.
53. Popovic-Todorovic B, Loft A, Bredkjaer HE, et al. A prospective randomized clinical trial comparing an individual dose of recombinant FSH based on predictive factors versus a ‘standard’ dose of 150 IU/day in ‘standard’ patients undergoing IVF/ICSI treatment. *Hum Reprod.* 2003;18(11):2275–82.
54. Howles CM, Saunders H, Alam V, Engrand P. Predictive factors and a corresponding treatment algorithm for controlled ovarian stimulation in patients treated with recombinant human follicle stimulating hormone (follitropin alfa) during assisted reproduction technology (ART) procedures. An analysis of 1378 patients. *Curr Med Res Opin.* 2006;22(5):907–18.
55. La Marca A, Sunkara SK. Individualization of controlled ovarian stimulation in IVF using ovarian reserve markers: From theory to practice. *Hum Reprod Update.* 2014;20(1):124–40.
56. Ubaldi FM, Rienzi L, Ferrero S, et al. Management of poor responders in IVF. *Reprod Biomed Online.* 2005;10(2):235–46.
57. Pandian Z, McTavish AR, Aucott L, et al. Interventions for ‘poor responders’ to controlled ovarian hyper stimulation (COH) in in-vitro fertilisation (IVF). *Cochrane Data Syst Rev.* 2010(1):CD004379.
58. Keay SD, Liversedge NH, Mathur RS, Jenkins JM. Assisted conception following poor ovarian response to gonadotrophin stimulation. *Br J Obstet Gynaecol.* 1997;104(5):521–7.
59. Karande V, Gleicher N. A rational approach to the management of low responders in in-vitro fertilization. *Hum Reprod.* 1999;14(7):1744–8.
60. Fasouliotis SJ, Simon A, Laufer N. Evaluation and treatment of low responders in assisted reproductive technology: A challenge to meet. *J Assist Reprod Genet.* 2000;17(7):357–73.
61. Tarlatzis BC, Zepiridis L, Grimbizis G, Bontis J. Clinical management of low ovarian response to stimulation for IVF: A systematic review. *Hum Reprod Update.* 2003;9(1):61–76.
62. Ben-Rafael Z, Strauss JF, Mastrianni L Jr., Flickinger GL. Differences in ovarian stimulation in human menopausal gonadotropin treated woman may be related to follicle-stimulating hormone accumulation. *Fertil Steril.* 1986;46(4):586–92.
63. Ben-Rafael Z, Benadiva CA, Ausmanas M, et al. Dose of human menopausal gonadotropin influences the outcome of an in vitro fertilization program. *Fertil Steril.* 1987;48(6):964–8.
64. Hofmann GE, Toner JP, Muasher SJ, Jones GS. High-dose follicle-stimulating hormone (FSH) ovarian stimulation in low-responder patients for in vitro fertilization. *J In Vitro Fert Embryo Transf.* 1989;6(5):285–9.
65. Berkkanoglu M, Ozgur K. What is the optimum maximal gonadotropin dosage used in microdose flare-up cycles in poor responders? *Fertil Steril.* 2010;94(2):662–5.
66. Lefebvre J, Antaki R, Kadoch IJ, et al. 450 IU versus 600 IU gonadotropin for controlled ovarian stimulation in poor responders: A randomized controlled trial. *Fertil Steril.* 2015;104(6):1419–25.
67. van Hooff MH, Alberda AT, Huisman GJ, et al. Doubling the human menopausal gonadotrophin dose in the course of an in-vitro fertilization treatment cycle in low responders: A randomized study. *Hum Reprod.* 1993;8(3):369–73.
68. Ovarian Stimulation T, Bosch E, Broer S, Griesinger G, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI(dagger). *Hum Reprod Open.* 2020;2020(2):hoa009.
69. Hughes EG, Fedorkow DM, Daya S, et al. The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: A meta-analysis of randomized controlled trials. *Fertil Steril.* 1992;58(5):888–96.
70. Daya S. Optimal protocol for gonadotropin-releasing hormone agonist use in ovarian stimulation. In: *In Vitro Fertilization and Assisted Reproduction.* Gomel V, Cheung PCK (eds.). Bologna, Italy: Mondadori Editore, pp. 405–15, 1997.
71. Ben-Rafael Z, Bider D, Dan U, et al. Combined gonadotropin releasing hormone agonist/human menopausal gonadotropin therapy (GnRH-a/hMG) in normal, high, and poor responders to hMG. *J In Vitro Fert Embryo Transf.* 1991;8(1):33–6.
72. Chetkowski RJ, Rode RA, Burruel V, Nass TE. The effect of pituitary suppression and the women’s age on embryo viability and uterine receptivity. *Fertil Steril.* 1991;56(6):1095–103.
73. Lessing JB, Cohen JR, Yovel I, et al. Atypical response to luteinizing hormone-releasing hormone (LH-RH) agonist (suprefact nasal) in induction of ovulation in in vitro fertilization (IVF). *J In Vitro Fert Embryo Transf.* 1991;8(6):314–6.
74. Ben-Nun I, Jaffe R, Goldberger S, et al. Complete ovarian unresponsiveness to hMG stimulation after prolonged GnRH analogue administration. *Gynecol Endocrinol.* 1990;4(3):151–5.
75. Davis OK, Rosenwaks Z. The ovarian factor in assisted reproductive technology. In: *The Ovary.* Adashi E, Leung P (eds.). New York: Raven Press, pp. 545–60, 1993.
76. Meldrum DR, Tsao Z, Monroe SE, et al. Stimulation of LH fragments with reduced bioactivity following GnRH agonist administration in women. *J Clin Endocrinol Metab.* 1984;58(4):755–7.

77. Cedars MI, Surey E, Hamilton F, et al. Leuprolide acetate lowers circulating bioactive luteinizing hormone and testosterone concentrations during ovarian stimulation for oocyte retrieval. *Fertil Steril.* 1990;53(4):627–31.
78. Dahl KD, Pavlou SN, Kovacs WJ, Hsueh AJ. The changing ratio of serum bioactive to immunoreactive follicle-stimulating hormone in normal men following treatment with a potent gonadotropin releasing hormone antagonist. *J Clin Endocrinol Metab.* 1986;63(3):792–4.
79. Scott RT, Neal GS, Illions EH, et al. The duration of leuprolide acetate administration prior to ovulation induction does not impact ovarian responsiveness to exogenous gonadotropins. *Fertil Steril.* 1993;60(2):247–53.
80. Kowalik A, Barmat L, Damario M, et al. Ovarian estradiol production in vivo. Inhibitory effect of leuprolide acetate. *J Reprod Med.* 1998;43(5):413–7.
81. Latouche J, Crumeyrolle-Arias M, Jordan D, et al. GnRH receptors in human granulosa cells: Anatomical localization and characterization by autoradiographic study. *Endocrinology.* 1989;125(3):1739–41.
82. Sandow J, Stoeckemann K, Jerabek-Sandow G. Pharmacokinetics and endocrine effects of slow release formulations of LHRH analogues. *J Steroid Biochem Mol Biol.* 1990;37(6):925–31.
83. Janssens RM, Lambalk CB, Vermeiden JP, et al. Dose-finding study of triptorelin acetate for prevention of a premature LH surge in IVF: A prospective, randomized, double-blind, placebo-controlled study. *Hum Reprod.* 2000;15(11):2333–40.
84. Feldberg D, Farhi J, Ashkenazi J, et al. Minidose gonadotropin-releasing hormone agonist is the treatment of choice in poor responders with high follicle-stimulating hormone levels. *Fertil Steril.* 1994;62(2):343–6.
85. Olivennes F, Righini C, Fanchin R, et al. A protocol using a low dose of gonadotrophin-releasing hormone agonist might be the best protocol for patients with high follicle-stimulating hormone concentrations on day 3. *Hum Reprod.* 1996;11(6):1169–72.
86. Weissman A, Farhi J, Royburt M, et al. Prospective evaluation of two stimulation protocols for low responders who were undergoing in vitro fertilization-embryo transfer. *Fertil Steril.* 2003;79(4):886–92.
87. Keltz MD, Jones EE, Duleba AJ, et al. Baseline cyst formation after luteal phase gonadotropin-releasing hormone agonist administration is linked to poor in vitro fertilization outcome. *Fertil Steril.* 1995;64(3):568–72.
88. Session DR, Saad AH, Salmansohn DD, Kelly AC. Ovarian activity during follicular-phase down regulation in in vitro fertilization is associated with advanced maternal age and a high recurrence rate in subsequent cycles. *J Assist Reprod Genet.* 1995;12(5):301–4.
89. Margalioth EJ, Kafka I, Friedler S, et al. eds. The incidence of ovarian cysts formation following GnRH analog treatment in IVF cycles is related to serum progesterone on the day of analog treatment initiation. 7th Annual Meeting ESHRE, Paris, France; 1991 June 28–30, 1991.
90. Aston K, Arthur I, Masson GM, Jenkins JM. Progestogen therapy and prevention of functional ovarian cysts during pituitary desensitisation with GnRH agonists. *Br J Obstet Gynaecol.* 1995;102(10):835–7.
91. Ditkoff EC, Sauer MV. A combination of norethindrone acetate and leuprolide acetate blocks the gonadotrophin-releasing hormone agonistic response and minimizes cyst formation during ovarian stimulation. *Hum Reprod.* 1996;11(5):1035–7.
92. Engmann L, Maconochie N, Bekir J, Tan SL. Progestogen therapy during pituitary desensitization with gonadotropin-releasing hormone agonist prevents functional ovarian cyst formation: A prospective, randomized study. *AJOG.* 1999;181(3):576–82.
93. Sunkara SK, Coomarasamy A, Faris R, et al. Long gonadotropin-releasing hormone agonist versus short agonist versus antagonist regimens in poor responders undergoing in vitro fertilization: A randomized controlled trial. *Fertil Steril.* 2014;101(1):147–53.
94. Smitz J, Devroey P, Camus M, et al. Inhibition of gonadotropic and ovarian function by intranasal administration of D-Ser (TBU)6-EA10-LHRH in normo-ovulatory women and patients with polycystic ovary disease. *J Endocrinol Invest.* 1988;11(9):647–52.
95. Calogero AE, Macchi M, Montanini V, et al. Dynamics of plasma gonadotropin and sex steroid release in polycystic ovarian disease after pituitary-ovarian inhibition with an analog of gonadotropin-releasing hormone. *J Clin Endocrinol Metab.* 1987;64(5):980–5.
96. Sungurtekin U, Jansen RP. Profound luteinizing hormone suppression after stopping the gonadotropin-releasing hormone-agonist leuprolide acetate. *Fertil Steril.* 1995;63(3):663–5.
97. Corson SL, Batzer FR, Gocial B, et al. Leuprolide acetate-prepared in vitro fertilization-gamete intrafallopian transfer cycles: Efficacy versus controls and cost analysis. *Fertil Steril.* 1992;57(3):601–5.
98. Pantos K, Meimeth-Damianaki T, Vaxevanoglou T, Kapetanakis E. Prospective study of a modified gonadotropin-releasing hormone agonist long protocol in an in vitro fertilization program. *Fertil Steril.* 1994;61(4):709–13.
99. Simons AH, Roelofs HJ, Schmoutziger AP, et al. Early cessation of triptorelin in in vitro fertilization: A double-blind, randomized study. *Fertil Steril.* 2005;83(4):889–96.
100. Beckers NG, Laven JS, Eijkemans MJ, Fauser BC. Follicular and luteal phase characteristics following early cessation of gonadotrophin-releasing hormone agonist during ovarian stimulation for in-vitro fertilization. *Hum Reprod.* 2000;15(1):43–9.
101. Fujii S, Sagara M, Kudo H, et al. A prospective randomized comparison between long and discontinuous-long protocols of gonadotropin-releasing hormone agonist for in vitro fertilization. *Fertil Steril.* 1997;67(6):1166–8.
102. Cedrin-Durnerin I, Bidart JM, Robert P, et al. Consequences on gonadotrophin secretion of an early discontinuation of gonadotrophin-releasing hormone agonist administration in short-term protocol for in-vitro fertilization. *Hum Reprod.* 2000;15(5):1009–14.
103. Detti L, Williams DB, Robins JC, et al. A comparison of three downregulation approaches for poor responders undergoing in vitro fertilization. *Fertil Steril.* 2005;84(5):1401–5.
104. Faber BM, Mayer J, Cox B, et al. Cessation of gonadotropin-releasing hormone agonist therapy combined with high-dose gonadotropin stimulation yields favorable pregnancy results in low responders. *Fertil Steril.* 1998;69(5):826–30.
105. Wang PT, Lee RK, Su JT, et al. Cessation of low-dose gonadotropin-releasing hormone agonist therapy followed by high-dose gonadotropin stimulation yields a favorable ovarian response in poor responders. *J Assist Reprod Genet.* 2002;19(1):1–6.
106. Schachter M, Friedler S, Raziel A, et al. Improvement of IVF outcome in poor responders by discontinuation of GnRH analogue during the gonadotropin stimulation phase—a function of improved embryo quality. *J Assist Reprod Genet.* 2001;18(4):197–204.
107. Pinkas H, Orvieto R, Avrech OM, et al. Gonadotropin stimulation following GnRH-a priming for poor responders in in vitro fertilization-embryo transfer programs. *Gynecol Endocrinol.* 2000;14(1):11–4.
108. Dirnfeld M, Fruchter O, Yshai D, et al. Cessation of gonadotropin-releasing hormone analogue (GnRH-a) upon down-regulation versus conventional long GnRH-a protocol in poor responders undergoing in vitro fertilization. *Fertil Steril.* 1999;72(3):406–11.
109. Garcia-Velasco JA, Isaza V, Requena A, et al. High doses of gonadotrophins combined with stop versus non-stop protocol of GnRH analogue administration in low responder IVF patients: A prospective, randomized, controlled trial. *Hum Reprod.* 2000;15(11):2292–6.
110. Orvieto R, Kruchkovich J, Rabinson J, et al. Ultrashort gonadotropin-releasing hormone agonist combined with flexible multidose gonadotropin-releasing hormone antagonist for poor responders in in vitro fertilization/embryo transfer programs. *Fertil Steril.* 2008;90(1):228–30.

111. Orvieto R, Kirshenbaum M, Galiano V, et al. Stop GnRH-agonist combined with multiple-dose GnRH-antagonist protocol for patients with "Genuine" poor response undergoing controlled ovarian hyperstimulation for IVF. *Front Endocrinol.* 2020; 11:182.
112. Bider D, Ben-Rafael Z, Shalev J, et al. Pituitary and ovarian suppression rate after high dosage of gonadotropin-releasing hormone agonist. *Fertil Steril.* 1989;51(4):578–81.
113. Padilla SL, Dugan K, Maruschak V, et al. Use of the flare-up protocol with high dose human follicle stimulating hormone and human menopausal gonadotropins for in vitro fertilization in poor responders. *Fertil Steril.* 1996;65(4):796–9.
114. Garcia JE, Padilla SL, Bayati J, Baramki TA. Follicular phase gonadotropin-releasing hormone agonist and human gonadotropins: A better alternative for ovulation induction in in vitro fertilization. *Fertil Steril.* 1990;53(2):302–5.
115. Howles CM, Macnamee MC, Edwards RG. Short term use of an LHRH agonist to treat poor responders entering an in-vitro fertilization programme. *Hum Reprod.* 1987;2(8):655–6.
116. Katayama KP, Roesler M, Gunnarson C, et al. Short-term use of gonadotropin-releasing hormone agonist (leuprolide) for in vitro fertilization. *J In Vitro Fert Embryo Transf.* 1988;5(6):332–4.
117. Toth TL, Awwad JT, Veeck LL, et al. Suppression and flare regimens of gonadotropin-releasing hormone agonist. Use in women with different basal gonadotropin values in an in vitro fertilization program. *J Reprod Med.* 1996;41(5):321–6.
118. Karande V, Morris R, Rinehart J, et al. Limited success using the "flare" protocol in poor responders in cycles with low basal follicle-stimulating hormone levels during in vitro fertilization. *Fertil Steril.* 1997;67(5):900–3.
119. Brzyski RG, Muasher SJ, Drosch K, et al. Follicular atresia associated with concurrent initiation of gonadotropin-releasing hormone agonist and follicle-stimulating hormone for oocyte recruitment. *Fertil Steril.* 1988;50(6):917–21.
120. Gindoff PR, Hall JL, Stillman RJ. Ovarian suppression with leuprolide acetate: Comparison of luteal, follicular, and flare-up administration in controlled ovarian hyperstimulation for oocyte retrieval. *J In Vitro Fert Embryo Transf.* 1990;7(2): 94–7.
121. Anserini P, Magnasco A, Remorgida V, et al. Comparison of a blocking vs. a flare-up protocol in poor responders with a normal and abnormal clomiphene citrate challenge test. *Gynecol Endocrinol.* 1997;11(5):321–6.
122. Karacan M, Erkan H, Karabulut O, et al. Clinical pregnancy rates in an IVF program. Use of the flare-up protocol after failure with long regimens of GnRH-a. *J Reprod Med.* 2001;46(5):485–9.
123. San Roman GA, Surrey ES, Judd HL, Kerin JF. A prospective randomized comparison of luteal phase versus concurrent follicular phase initiation of gonadotropin-releasing hormone agonist for in vitro fertilization. *Fertil Steril.* 1992;58(4):744–9.
124. Hillier SG. Roles of follicle stimulating hormone and luteinizing hormone in controlled ovarian hyperstimulation. *Hum Reprod.* 1996;11(Suppl 3):113–21.
125. Gelety TJ, Pearlstone AC, Surrey ES. Short-term endocrine response to gonadotropin-releasing hormone agonist initiated in the early follicular, midluteal, or late luteal phase in normally cycling women. *Fertil Steril.* 1995;64(6):1074–80.
126. Cedrin-Durnerin I, Bulwa S, Herve F, et al. The hormonal flare-up following gonadotrophin-releasing hormone agonist administration is influenced by a progestogen pretreatment. *Hum Reprod.* 1996;11(9):1859–63.
127. al-Mizyen E, Sabatini L, Lower AM, et al. Does pretreatment with progestogen or oral contraceptive pills in low responders followed by the GnRHa flare protocol improve the outcome of IVF-ET? *J Assist Reprod Genet.* 2000;17(3):140–6.
128. Bstandig B, Cedrin-Durnerin I, Hugues JN. Effectiveness of low dose of gonadotropin releasing hormone agonist on hormonal flare-up. *J Assist Reprod Genet.* 2000;17(2):113–7.
129. Deaton JL, Baugess P, Huffman CS, Miller KA. Pituitary response to early follicular-phase mididose gonadotropin releasing hormone agonist (GnRHa) therapy: Evidence for a second flare. *J Assist Reprod Genet.* 1996;13(5):390–4.
130. Navot D, Rosenwaks Z, Anderson F, Hodgen GD. Gonadotropin-releasing hormone agonist-induced ovarian hyperstimulation: Low-dose Side effects in women and monkeys. *Fertil Steril.* 1991;55(6):1069–75.
131. Scott RT, Carey KD, Leland M, Navot D. Gonadotropin responsiveness to ultralow-dose leuprolide acetate administration in baboons. *Fertil Steril.* 1993;59(5):1124–8.
132. Scott RT, Navot D. Enhancement of ovarian responsiveness with microdoses of gonadotropin-releasing hormone agonist during ovulation induction for in vitro fertilization. *Fertil Steril.* 1994;61(5):880–5.
133. Schoolcraft W, Schlenker T, Gee M, et al. Improved controlled ovarian hyperstimulation in poor responder in vitro fertilization patients with a microdose follicle-stimulating hormone flare, growth hormone protocol. *Fert Steril.* 1997;67(1):93–7.
134. Surrey ES, Bower J, Hill DM, et al. Clinical and endocrine effects of a microdose GnRH agonist flare regimen administered to poor responders who are undergoing in vitro fertilization. *Fertil Steril.* 1998;69(3):419–24.
135. Leondires MP, Escalpés M, Segars JH, et al. Microdose follicular phase gonadotropin-releasing hormone agonist (GnRH-a) compared with luteal phase GnRH-a for ovarian stimulation at in vitro fertilization. *Fertil Steril.* 1999;72(6):1018–23.
136. Albano C, Felberbaum RE, Smitz J, et al. Ovarian stimulation with HMG: Results of a prospective randomized phase III European study comparing the luteinizing hormone-releasing hormone (LHRH)-antagonist cetrorelix and the LHRH-agonist buserelin. European Cetrorelix Study Group. *Hum Reprod.* 2000;15(3):526–31.
137. Howles CM. The place of gonadotrophin-releasing hormone antagonists in reproductive medicine. *Reprod Biomed Online.* 2002;4(Suppl 3):64–71.
138. Al-Inany HG, Youssef MA, Aboulghar M, et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Data Syst Rev.* 2011(5):CD001750.
139. Quigley MM. The use of ovulation-inducing agents in in-vitro fertilization. *Clin Obstet Gynecol.* 1984;27(4):983–92.
140. Lehmann F, Baban N, Webber B. Ovarian stimulation for in-vitro fertilization: Clomiphene and HMG. *Hum Reprod.* 1988;3(Suppl 2): 11–21.
141. Ronen J, Bosschieter J, Wiswedel K, et al. Ovulation induction for in vitro fertilisation using clomiphene citrate and low-dose human menopausal gonadotrophin. *Int J Fertil.* 1988;33(2): 120–2.
142. Tummon IS, Daniel SA, Kaplan BR, et al. Randomized, prospective comparison of luteal leuprolide acetate and gonadotropins versus clomiphene citrate and gonadotropins in 408 first cycles of in vitro fertilization. *Fertil Steril.* 1992;58(3):563–8.
143. Craft I, Gorgy A, Hill J, Menon D, Podsiadly B. Will GnRH antagonists provide new hope for patients considered 'difficult responders' to GnRH agonist protocols? *Hum Reprod.* 1999;14(12): 2959–62.
144. Nikolettos N, Al-Hasani S, Felberbaum R, et al. Gonadotropin-releasing hormone antagonist protocol: A novel method of ovarian stimulation in poor responders. *Eur J Obstet Gynecol Reprod Biol.* 2001;97(2):202–7.
145. Posada MN, Vlahos NP, Jurema MW, et al. Clinical outcome of using ganirelix acetate versus a 4-day follicular phase leuprolide acetate protocol in unselected women undergoing in vitro fertilization. *Fertil Steril.* 2003;80(1):103–10.
146. Mohamed KA, Davies WA, Allsopp J, Lashen H. Agonist "flare-up" versus antagonist in the management of poor responders undergoing in vitro fertilization treatment. *Fertil Steril.* 2005;83(2): 331–5.

147. Fasouliotis SJ, Laufer N, Sabbagh-Ehrlich S, et al. Gonadotropin-releasing hormone (GnRH)-antagonist versus GnRH-agonist in ovarian stimulation of poor responders undergoing IVF. *J Assist Reprod Genet.* 2003;20(11):455–60.
148. Copperman AB. Antagonists in poor-responder patients. *Fertil Steril.* 2003;80(Suppl 1):S16–24; discussion S32–4.
149. Shapiro D, Carter M, Mitchell-Leef D, Wininger D. Plateau or drop in estradiol (E2) on the day after initiation of the GnRH antagonist antagonist™ in in-vitro fertilization (IVF) treatment cycles does not affect pregnancy outcome. *Fertil Steril.* 2002;78(S1):S22–S3.
150. Akman MA, Erden HF, Tosun SB, et al. Addition of GnRH antagonist in cycles of poor responders undergoing IVF. *Hum Reprod.* 2000;15(10):2145–7.
151. Akman MA, Erden HF, Tosun SB, et al. Comparison of agonistic flare-up-protocol and antagonistic multiple dose protocol in ovarian stimulation of poor responders: Results of a prospective randomized trial. *Hum Reprod.* 2001;16(5):868–70.
152. De Placido G, Mollo A, Clarizia R, et al. Gonadotropin-releasing hormone (GnRH) antagonist plus recombinant luteinizing hormone vs. a standard GnRH agonist short protocol in patients at risk for poor ovarian response. *Fertil Steril.* 2006;85(1):247–50.
153. Demiroglu A, Gurgan T. Comparison of microdose flare-up and antagonist multiple-dose protocols for poor-responder patients: A randomized study. *Fertil Steril.* 2009;92(2):481–5.
154. Kahraman K, Berker B, Atabekoglu CS, et al. Microdose gonadotropin-releasing hormone agonist flare-up protocol versus multiple dose gonadotropin-releasing hormone antagonist protocol in poor responders undergoing intracytoplasmic sperm injection–embryo transfer cycle. *Fertil Steril.* 2009;91(6):2437–44.
155. Devesa M, Martinez F, Coroleu B, Tur R, et al. Poor prognosis for ovarian response to stimulation: Results of a randomised trial comparing the flare-up GnRH agonist protocol vs. the antagonist protocol. *Gynecol Endocrinol.* 2010;26(7):509–15.
156. Schmidt DW, Bremner T, Orris JJ, et al. A randomized prospective study of microdose leuprolide versus ganirelix in in vitro fertilization cycles for poor responders. *Fertil Steril.* 2005;83(5):1568–71.
157. Malmusi S, La Marca A, Giulini S, et al. Comparison of a gonadotropin-releasing hormone (GnRH) antagonist and GnRH agonist flare-up regimen in poor responders undergoing ovarian stimulation. *Fertil Steril.* 2005;84(2):402–6.
158. D'Amato G, Caroppo E, Pasquadibisceglie A, et al. A novel protocol of ovulation induction with delayed gonadotropin-releasing hormone antagonist administration combined with high-dose recombinant follicle-stimulating hormone and clomiphene citrate for poor responders and women over 35 years. *Fertil Steril.* 2004;81(6):1572–7.
159. Tazegul A, Gorkemli H, Ozdemir S, Aktan TM. Comparison of multiple dose GnRH antagonist and mididose long agonist protocols in poor responders undergoing in vitro fertilization: A randomized controlled trial. *Arch Gynecol Obstet.* 2008;278(5):467–72.
160. Marci R, Caserta D, Dolo V, et al. GnRH antagonist in IVF poor responder patients: Results of a randomized trial. *Reprod Biomed Online.* 2005;11(2):189–93.
161. Cheung LP, Lam PM, Lok IH, et al. GnRH antagonist versus long GnRH agonist protocol in poor responders undergoing IVF: A randomized controlled trial. *Hum Reprod.* 2005;20(3):616–21.
162. Frankfurter D, Dayal M, Dubey A, et al. Novel follicular-phase gonadotropin-releasing hormone antagonist stimulation protocol for in vitro fertilization in the poor responder. *Fertil Steril.* 2007;88(5):1442–5.
163. Berger BM, Ezcurra D, Alper MM. The agonist-antagonist protocol: A novel protocol for treating the poor responder [abstract]. *Fertil Steril.* 2004;82(Suppl 2):S126.
164. Fisch JD, Keskinen L, Sher G. Gonadotropin-releasing hormone agonist/antagonist conversion with estrogen priming in low responders with prior in vitro fertilization failure. *Fertil Steril.* 2008;89(2):342–7.
165. Nargund G, Fauser BC, Macklon NS, et al. The ISMAAR proposal on terminology for ovarian stimulation for IVF. *Hum Reprod.* 2007;22(11):2801–4.
166. Bassil S, Godin PA, Donnez J. Outcome of in-vitro fertilization through natural cycles in poor responders. *Hum Reprod.* 1999;14(5):1262–5.
167. Feldman B, Seidman DS, Levron J, et al. In vitro fertilization following natural cycles in poor responders. *Gynecol Endocrinol.* 2001;15(5):328–34.
168. Lindheim SR, Vidali A, Ditkoff E, et al. Poor responders to ovarian hyperstimulation may benefit from an attempt at natural-cycle oocyte retrieval. *J Assist Reprod Genetics.* 1997;14(3):174–6.
169. Bar-Hava I, Ferber A, Ashkenazi J, et al. Natural-cycle in vitro fertilization in women aged over 44 years. *Gynecol Endocrinol.* 2000;14(4):248–52.
170. Check ML, Check JH, Wilson C, et al. Outcome of in vitro fertilization-embryo transfer according to age in poor responders with elevated baseline serum follicle stimulation hormone using minimal or no gonadotropin stimulation. *Clin Exp Obstet Gynecol.* 2004;31(3):183–4.
171. Morgia F, Sbraccia M, Schimberni M, et al. A controlled trial of natural cycle versus microdose gonadotropin-releasing hormone analog flare cycles in poor responders undergoing in vitro fertilization. *Fertil Steril.* 2004;81(6):1542–7.
172. Papaleo E, De Santis L, Fusi F, et al. Natural cycle as first approach in aged patients with elevated follicle-stimulating hormone undergoing intracytoplasmic sperm injection: A pilot study. *Gynecol Endocrinol.* 2006;22(7):351–4.
173. Polyzos NP, Blockeel C, Verpoest W, et al. Live birth rates following natural cycle IVF in women with poor ovarian response according to the Bologna criteria. *Hum Reprod.* 2012;27(12):3481–6.
174. Pelinck MJ, Hoek A, Simons AH, Heineman MJ. Efficacy of natural cycle IVF: A review of the literature. *Hum Reprod Update.* 2002;8(2):129–39.
175. Paulson RJ, Sauer MV, Lobo RA. Addition of a gonadotropin releasing hormone (GnRH) antagonist and exogenous gonadotropins to unstimulated in vitro fertilization (IVF) cycles: Physiologic observations and preliminary experience. *J Assist Reprod Genet.* 1994;11(1):28–32.
176. Rongieres-Bertrand C, Olivennes F, Righini C, et al. Revival of the natural cycles in in-vitro fertilization with the use of A new gonadotrophin-releasing hormone antagonist (cetrorelix): A pilot study with minimal stimulation. *Hum Reprod.* 1999;14(3):683–8.
177. Pelinck MJ, Vogel NE, Arts EG, et al. Cumulative pregnancy rates after A maximum of nine cycles of modified natural cycle IVF and analysis of patient drop-out: A cohort study. *Hum Reprod.* 2007;22(9):2463–70.
178. Pelinck MJ, Vogel NE, Hoek A, et al. Cumulative pregnancy rates after three cycles of minimal stimulation IVF and results according to subfertility diagnosis: A multicentre cohort study. *Hum Reprod.* 2006;21(9):2375–83.
179. Pelinck MJ, Vogel NE, Hoek A, et al. Minimal stimulation IVF with late follicular phase administration of the GnRH antagonist cetrorelix and concomitant substitution with recombinant FSH: A pilot study. *Hum Reprod.* 2005;20(3):642–8.
180. Kolibianakis E, Zikopoulos K, Camus M, et al. Modified natural cycle for IVF does not offer a realistic chance of parenthood in poor responders with high day 3 FSH levels, as a last resort prior to oocyte donation. *Hum Reprod.* 2004;19(11):2545–9.
181. Elizur SE, Aslan D, Shulman A, et al. Modified natural cycle using GnRH antagonist can be an optional treatment in poor responders undergoing IVF. *J Assist Reprod Genet.* 2005;22(2):75–9.
182. Kim CH, Kim SR, Cheon YP, et al. Minimal stimulation using gonadotropin-releasing hormone (GnRH) antagonist and recombinant human follicle-stimulating hormone versus GnRH antagonist multiple-dose protocol in low responders undergoing in vitro fertilization/intracytoplasmic sperm injection. *Fertil Steril.* 2009;92(6):2082–4.

183. Kadoch IJ, Phillips SJ, Bissonnette F. Modified natural-cycle in vitro fertilization should be considered as the first approach in young poor responders. *Fertil Steril.* 2009;96(5):1066–8.
184. Kadoch IJ, Al-Khaduri M, Phillips SJ, et al. Spontaneous ovulation rate before oocyte retrieval in modified natural cycle IVF with and without indomethacin. *Reprod Biomed Online.* 2008;16(2):245–9.
185. Schimberni M, Morgia F, Colabianchi J, et al. Natural-cycle in vitro fertilization in poor responder patients: A survey of 500 consecutive cycles. *Fertil Steril.* 2009;92(4):1297–301.
186. Castelo Branco A, Achour-Frydman N, Kadoch J, et al. In vitro fertilization and embryo transfer in seminatural cycles for patients with ovarian aging. *Fertil Steril.* 2005;84(4):875–80.
187. Segawa T, Yelian Y, Kato K, et al. Natural cycle IVF is an excellent treatment option for women with advanced age. *Fertil Steril.* 2009;92(3 Suppl):S54.
188. Ellenbogen A, Gidoni Y, Atamna R, et al. Last chance before egg donation: Modified natural cycle in vitro fertilization in poor responder patients; The role of follicle diameter on The day of hCG administration in order to improve results. *Fertil Steril.* 2009;92(3 Suppl):S162.
189. Ellenbogen A, Michaeli M, Ballas S. Egg collection with a double lumen needle in poor responder patients undergoing in vitro fertilization treatment. *Fertil Steril.* 2003;80(Suppl 3):127.
190. Méndez Lozano DH, Brum Scheffer J, et al. Optimal reproductive competence of oocytes retrieved through follicular flushing in minimal stimulation IVF. *Reprod Biomed Online.* 2008;16(1):119–23.
191. Mendez Lozano DH, Fanchin R, Chevalier N, et al. [The follicular flushing duplicate the pregnancy rate on semi natural cycle IVF]. *J Gynecol Obstet Biol Reprod (Paris).* 2007;36(1):36–41.
192. Levens ED, Whitcomb BW, Payson MD, Larsen FW. Ovarian follicular flushing among low-responding patients undergoing assisted reproductive technology. *Fertil Steril.* 2009;91(4 Suppl):1381–4.
193. Mochtar MH, Van der V, Ziech M, van Wely M. Recombinant luteinizing hormone (rLH) for controlled ovarian hyperstimulation in assisted reproductive cycles. *Cochrane Data Syst Rev.* 2007(2):CD005070.
194. Simoni M, Nieschlag E, Gromoll J. Isoforms and single nucleotide polymorphisms of the FSH receptor gene: Implications for human reproduction. *Hum Reprod Update.* 2002;8(5):413–21.
195. Flack MR, Froehlich J, Bennet AP, et al. Site-directed mutagenesis defines the individual roles of the glycosylation sites on follicle-stimulating hormone. *J Biol Chem.* 1994;269(19):14015–20.
196. Howles CM. Genetic engineering of human FSH (Gonal-F). *Hum Reprod Update.* 1996;2(2):172–91.
197. Bassett RM, Driebergen R. Continued improvements in the quality and consistency of follitropin alfa, recombinant human FSH. *Reprod Biomed Online.* 2005;10(2):169–77.
198. Aittomaki K, Herva R, Stenman UH, et al. Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene. *J Clin Endocrinol Metab.* 1996;81(10):3722–6.
199. Touraine P, Beau I, Gougeon A, et al. New natural inactivating mutations of the follicle-stimulating hormone receptor: Correlations between receptor function and phenotype. *Mol Endocrinol.* 1999;13(11):1844–54.
200. Perez Mayorga M, Gromoll J, Behre HM, et al. Ovarian response to follicle-stimulating hormone (FSH) stimulation depends on the FSH receptor genotype. *J Clin Endocrinol Metab.* 2000;85(9):3365–9.
201. Jun JK, Yoon JS, Ku SY, et al. Follicle-stimulating hormone receptor gene polymorphism and ovarian responses to controlled ovarian hyperstimulation for IVF-ET. *J Hum Genet.* 2006;51(8):665–70.
202. Greb RR, Grieshaber K, Gromoll J, et al. A common single nucleotide polymorphism in exon 10 of the human follicle stimulating hormone receptor is a major determinant of length and hormonal dynamics of the menstrual cycle. *J Clin Endocrinol Metab.* 2005;90(8):4866–72.
203. Behre HM, Greb RR, Mempel A, et al. Significance of A common single nucleotide polymorphism in exon 10 of the follicle-stimulating hormone (FSH) receptor gene for the ovarian response to FSH: A pharmacogenetic approach to controlled ovarian hyperstimulation. *Pharmacogenet Genomics.* 2005;15(7):451–6.
204. Achrekar SK, Modi DN, Desai SK, et al. Poor ovarian response to gonadotrophin stimulation is associated with FSH receptor polymorphism. *Reprod Biomed Online.* 2009;18(4):509–15.
205. Sudo S, Kudo M, Wada S, et al. Genetic and functional analyses of polymorphisms in the human FSH receptor gene. *Mol Hum Reprod.* 2002;8(10):893–9.
206. Laven JS, Mulders AG, Suryandari DA, et al. Follicle-stimulating hormone receptor polymorphisms in women with normogonadotropic anovulatory infertility. *Fertil Steril.* 2003;80(4):986–92.
207. de Castro F, Moron FJ, Montoro L, et al. Pharmacogenetics of controlled ovarian hyperstimulation. *Pharmacogenomics.* 2005;6(6):629–37.
208. Daelemans C, Smits G, de Maertelaer V, et al. Prediction of severity of symptoms in iatrogenic ovarian hyperstimulation syndrome by follicle-stimulating hormone receptor Ser680Asn polymorphism. *J Clin Endocrinol Metab.* 2004;89(12):6310–5.
209. Choi D, Lee EY, Yoon S, et al. Clinical correlation of cyclin D2 mRNA expression in human luteinized granulosa cells. *J Assist Reprod Genet.* 2000;17(10):574–9.
210. Pirtea P, de Ziegler D, Marin D, et al. Gonadotropin receptor polymorphisms (FSHR N680S and LHCGR N312S) are not predictive of clinical outcome and live birth in assisted reproductive technology. *Fertil Steril.* 2022;118(3):494–503.
211. Bosch E, Ezcurra D. Individualised controlled ovarian stimulation (iCOS): Maximising success rates for assisted reproductive technology patients. *Reprod Biol Endocrinol.* 2011;9:82.
212. Nardo LG, Fleming R, Howles CM, et al. Conventional ovarian stimulation no longer exists: Welcome to the age of individualized ovarian stimulation. *Reprod Biomed Online.* 2011;23(2):141–8.
213. Olivennes F, Borini A, Germond M, et al, eds. Optimising ovarian stimulation in ART using a novel FSH dose algorithm and follitropin alfa filled by mass: results of the CONSORT multinational study. 23rd Annual Meeting of the ESHRE, Lyon, France, 1–4 July 2007.
214. Balasch J, Miro F, Burzaco I, et al. The role of luteinizing hormone in human follicle development and oocyte fertility: Evidence from in-vitro fertilization in a woman with long-standing hypogonadotropic hypogonadism and using recombinant human follicle stimulating hormone. *Hum Reprod.* 1995;10(7):1678–83.
215. Hull M, Corrigan E, Piazza A, Loumaye E. Recombinant human luteinising hormone: An effective new gonadotropin preparation. *Lancet.* 1994;344(8918):334–5.
216. De Placido G, Alviggi C, Mollo A, et al. Effects of recombinant LH (rLH) supplementation during controlled ovarian hyperstimulation (COH) in normogonadotropic women with an initial inadequate response to recombinant FSH (rFSH) after pituitary downregulation. *Clin Endocrinol.* 2004;60(5):637–43.
217. Howles CM. Role of LH and FSH in ovarian function. *Mol Cell Endocrinol.* 2000;161(1-2):25–30.
218. Chappel SC, Howles C. Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. *Hum Reprod.* 1991;6(9):1206–12.
219. Hillier SG. Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Hum Reprod.* 1994;9(2):188–91.
220. Balasch J, Fabregues F. Is luteinizing hormone needed for optimal ovulation induction? *Curr Opin Obstet Gynecol.* 2002;14(3):265–74.
221. Shoham Z. Treatment of female infertility with recombinant human luteinising hormone: Is there a benefit over other available drugs? *Expert Opin Pharmacother.* 2003;4(11):1985–94.

222. Huirne JA, van Loenen AC, Schats R, et al. Dose-finding study of daily GnRH antagonist for the prevention of premature LH surges in IVF/ICSI patients: Optimal changes in LH and progesterone for clinical pregnancy. *Hum Reprod.* 2005;20(2):359–67.
223. Haavisto AM, Pettersson K, Bergendahl M, et al. Occurrence and biological properties of a common genetic variant of luteinizing hormone. *J Clin Endocrinol Metab.* 1995;80(4):1257–63.
224. Alviggi C, Clarizia R, Pettersson K, et al. Suboptimal response to GnRH α long protocol is associated with a common LH polymorphism. *Reprod Biomed Online.* 2009;18(1):9–14.
225. Takahashi K, Kurioka H, Ozaki T, et al. Increased prevalence of luteinizing hormone beta-subunit variant in Japanese infertility patients. *Hum Reprod.* 1998;13(12):3338–44.
226. Takahashi K, Ozaki T, Okada M, et al. Increased prevalence of luteinizing hormone beta-subunit variant in patients with premature ovarian failure. *Fertil Steril.* 1999;71(1):96–101.
227. The European Recombinant Human LH Study Group. Recombinant human luteinizing hormone (LH) to support recombinant human follicle-stimulating hormone (FSH)-induced follicular development in LH- and FSH-deficient anovulatory women: A dose-finding study. *J Clin Endocrinol Metab.* 1998;83(5):1507–14.
228. Burgues S, Spanish Collaborative Group on Female Hypogonadotropic Hypogonadism. The effectiveness and safety of recombinant human LH to support follicular development induced by recombinant human FSH in WHO group I anovulation: Evidence from a multicentre study in Spain. *Hum Reprod.* 2001;16(12):2525–32.
229. Loumaye E, Engrand P, Shoham Z, et al. Clinical evidence for an LH ‘ceiling’ effect induced by administration of recombinant human LH during the late follicular phase of stimulated cycles in world health organization type I and type II anovulation. *Hum Reprod.* 2003;18(2):314–22.
230. Hugues JN, Soussis J, Calderon I, et al. Does the addition of recombinant LH in WHO group II anovulatory women over-responding to FSH treatment reduce the number of developing follicles? A dose-finding study. *Hum Reprod.* 2005;20(3):629–35.
231. Melo P, Eapen A, Chung Y, et al. O-009° controlled ovarian stimulation (COS) protocols for assisted reproduction: A Cochrane systematic review and network meta-analysis. *Hum Reprod.* 2022;37(Supplement_1):deac104.009.
232. Foong SC, Abbott DH, Lesnick TG, et al. Diminished intrafollicular estradiol levels in in vitro fertilization cycles from women with reduced ovarian response to recombinant human follicle-stimulating hormone. *Fertil Steril.* 2005;83(5):1377–83.
233. Tesarik J, Mendoza C. Nongenomic effects of 17 beta-estradiol on maturing human oocytes: Relationship to oocyte developmental potential. *J Clin Endocrinol Metab.* 1995;80(4):1438–43.
234. Mason HD, Martikainen H, Beard RW, et al. Direct gonadotrophic effect of growth hormone on oestradiol production by human granulosa cells in vitro. *J Endocrinol.* 1990;126(3):R1–4.
235. Lanzone A, Fortini A, Fulghesu AM, et al. Growth hormone enhances estradiol production follicle-stimulating hormone-induced in the early stage of the follicular maturation. *Fertil Steril.* 1996;66(6):948–53.
236. Mendoza C, Ruiz-Requena E, Ortega E, et al. Follicular fluid markers of oocyte developmental potential. *Hum Reprod.* 2002;17(4):1017–22.
237. Tesarik J, Mendoza C. Direct non-genomic effects of follicular steroids on maturing human oocytes: Oestrogen versus androgen antagonism. *Hum Reprod Update.* 1997;3(2):95–100.
238. Murdoch WJ, Van Kirk EA. Estrogenic upregulation of DNA polymerase beta in oocytes of preovulatory ovine follicles. *Mol Reprod Dev.* 2001;58(4):417–23.
239. Zelinski-Wooten MB, Hess DL, Baughman WL, et al. Administration of an aromatase inhibitor during the late follicular phase of gonadotropin-treated cycles in rhesus monkeys: Effects on follicle development, oocyte maturation, and subsequent luteal function. *J Clin Endocrinol Metab.* 1993;76(4):988–95.
240. Lisi F, Rinaldi L, Fishel S, Lisi R, et al. Use of recombinant FSH and recombinant LH in multiple follicular stimulation for IVF: A preliminary study. *Reprod Biomed Online.* 2001;3(3):190–4.
241. De Placido G, Alviggi C, Perino A, et al. Recombinant human LH supplementation versus recombinant human FSH (rFSH) step-up protocol during controlled ovarian stimulation in normogonadotropic women with initial inadequate ovarian response to rFSH. A multicentre, prospective, randomized controlled trial. *Hum Reprod.* 2005;20(2):390–6.
242. Ferraretti AP, Gianaroli L, Magli MC, et al. Exogenous luteinizing hormone in controlled ovarian hyperstimulation for assisted reproduction techniques. *Fertil Steril.* 2004;82(6):1521–6.
243. Richards JS, Jahnson T, Hedin L, et al. Ovarian follicular development: From physiology to molecular biology. *Recent Prog Horm Res.* 1987;43:231–76.
244. Humaidan P, Chin W, Rogoff D, et al. Efficacy and safety of follitropin alfa/lutropin alfa in ART: A randomized controlled trial in poor ovarian responders. *Hum Reprod.* 2017;32(3):544–55.
245. Esteves SC, Yarali H, Vuong LN, et al. Cumulative delivery rate per aspiration IVF/ICSI cycle in POSEIDON patients: A real-world evidence study of 9073 patients. *Hum Reprod.* 2021;36(8):2157–69.
246. Robertson DM, Burger HG. Reproductive hormones: Ageing and the perimenopause. *Acta Obstet Gynecol Scand.* 2002;81(7):612–6.
247. Piltonen T, Koivunen R, Ruokonen A, Tapanainen JS. Ovarian age-related responsiveness to human chorionic gonadotropin. *J Clin Endocrinol Metab.* 2003;88(7):3327–32.
248. Vihko KK, Kujansuu E, Morsky P, Huhtaniemi I, Punnonen R. Gonadotropins and gonadotropin receptors during the perimenopause. *Eur J Endocrinol.* 1996;134(3):357–61.
249. Kim YK, Wasser SK, Fujimoto VY, et al. Utility of follicle stimulating hormone (FSH), luteinizing hormone (LH), oestradiol and FSH:LH ratio in predicting reproductive age in normal women. *Hum Reprod.* 1997;12(6):1152–5.
250. Mitchell R, Hollis S, Rothwell C, Robertson WR. Age related changes in the pituitary-testicular axis in normal men: Lower serum testosterone results from decreased bioactive LH drive. *Clin Endocrinol.* 1995;42(5):501–7.
251. Huhtaniemi IT, Pettersson KS. Alterations in gonadal steroidogenesis in individuals expressing a common genetic variant of luteinizing hormone. *J Steroid Biochem Mol Biol.* 1999;69(1–6):281–5.
252. De Placido G, Mollo A, Alviggi C, et al. Rescue of IVF cycles by HMG in pituitary down-regulated normogonadotropic young women characterized by a poor initial response to recombinant FSH. *Hum Reprod.* 2001;16(9):1875–9.
253. Loutradis D, Drakakis P, Milungos S, et al. Alternative approaches in the management of poor response in controlled ovarian hyperstimulation (COH). *Ann N Y Acad Sci.* 2003;997:112–9.
254. Gomez-Palomares JL, Acevedo-Martin B, Andres L, et al. LH improves early follicular recruitment in women over 38 years old. *Reprod Biomed Online.* 2005;11(4):409–14.
255. Conforti A, Esteves SC, Humaidan P, et al. Recombinant human luteinizing hormone co-treatment in ovarian stimulation for assisted reproductive technology in women of advanced reproductive age: A systematic review and meta-analysis of randomized controlled trials. *Reprod Biol Endocrinol.* 2021;19(1):91.
256. Bosch E, Labarta E, Crespo J, et al. Impact of luteinizing hormone administration on gonadotropin-releasing hormone Antagonist cycles: An age-adjusted Analysis. *Fertil Steril.* 2011;95(3):1031–6.
257. Younis JS, Izhaki I, Ben-Ami M. The effect of LH supplementation to the GnRH antagonist protocol in advanced reproductive ageing women: A prospective randomized controlled study. *Clin Endocrinol.* 2016;84(1):99–106.
258. Vuong TN, Phung HT, Ho MT. Recombinant follicle-stimulating hormone and recombinant luteinizing hormone versus recombinant follicle-stimulating hormone alone during GnRH antagonist ovarian stimulation in patients aged >/=35 years: A randomized controlled trial. *Hum Reprod.* 2015;30(5):1188–95.

259. Alviggi C, Mollo A, Clarizia R, De Placido G. Exploiting LH in ovarian stimulation. *Reprod Biomed Online*. 2006;12(2):221–33.
260. Nilsson C, Pettersson K, Millar RP, et al. Worldwide frequency of a common genetic variant of luteinizing hormone: An International Collaborative Research Group. *Fertil Steril*. 1997;67(6):998–1004.
261. de Castro F, Moron FJ, Montoro L, et al. Human controlled ovarian hyperstimulation outcome is a polygenic trait. *Pharmacogenetics*. 2004;14(5):285–93.
262. Andersen AN, Goossens V, Ferraretti AP, et al. Assisted reproductive technology in europe, 2004: Results generated from European registers by ESHRE. *Hum Reprod*. 2008;23(4):756–71.
263. Loutradis D, Elsheikh A, Kallianidis K, et al. Results of controlled ovarian stimulation for ART in poor responders according to the short protocol using different gonadotrophins combinations. *Arch Gynecol Obstet*. 2004;270(4):223–6.
264. Laml T, Obruca A, Fischl F, Huber JC. Recombinant luteinizing hormone in ovarian hyperstimulation after stimulation failure in normogonadotropic women. *Gynecol Endocrinol*. 1999;13(2):98–103.
265. Ferrari B, Barusi L, Coppola F. Clinical and endocrine effects of ovulation induction with FSH and hCG supplementation in low responders in the midfollicular phase. A pilot study. *J Reprod Med*. 2002;47(2):137–43.
266. Griesinger G, Shapiro DB, Kolibianakis EM, et al. No association between endogenous LH and pregnancy in a GnRH antagonist protocol: Part II, recombinant FSH. *Reprod Biomed Online*. 2011;23(4):457–65.
267. Chung K, Krey L, Katz J, Noyes N. Evaluating the role of exogenous luteinizing hormone in poor responders undergoing in vitro fertilization with gonadotropin-releasing hormone antagonists. *Fertil Steril*. 2005;84(2):313–8.
268. Berker B, Sukur YE, Ozdemir EU, et al. Human menopausal gonadotropin commenced on early follicular period increases live birth rates in POSEIDON group 3 and 4 poor responders. *Reprod Sci*. 2021;28(2):488–94.
269. Barrenetxea G, Agirrekoia JA, Jimenez MR. Ovarian response and pregnancy outcome in poor-responder women: A randomized controlled trial on the effect of luteinizing hormone supplementation on in vitro fertilization cycles. *Fertil Steril*. 2007;89(3):546–53.
270. Mochtar MH, Danhof NA, Ayeleke RO, et al. Recombinant luteinizing hormone (rLH) and recombinant follicle stimulating hormone (rFSH) for ovarian stimulation in IVF/ICSI cycles. *Cochrane Data Syst Rev*. 2017;5:CD005070.
271. Gonen Y, Jacobson W, Casper RF. Gonadotropin suppression with oral contraceptives before in vitro fertilization. *Fertil Steril*. 1990;53(2):282–7.
272. Biljan MM, Mahutte NG, Dean N, et al. Effects of pretreatment with an oral contraceptive on the time required to achieve pituitary suppression with gonadotropin-releasing hormone analogues and on subsequent implantation and pregnancy rates. *Fertil Steril*. 1998;70(6):1063–9.
273. Lindheim SR, Barad DH, Witt B, et al. Short-term gonadotropin suppression with oral contraceptives benefits poor responders prior to controlled ovarian hyperstimulation. *J Assist Reprod Genet*. 1996;13(9):745–7.
274. Kovacs P, Barg PE, Witt BR. Hypothalamic-pituitary suppression with oral contraceptive pills does not improve outcome in poor responder patients undergoing in vitro fertilization-embryo transfer cycles. *J Assist Reprod Genet*. 2001;18(7):391–4.
275. Fanchin R, Mendez Lozano DH, et al. Hormonal manipulations in the luteal phase to coordinate subsequent antral follicle growth during ovarian stimulation. *Reprod Biomed Online*. 2005;10(6):721–8.
276. Fanchin R, Salomon L, Castelo-Branco A, et al. Luteal estradiol pre-treatment coordinates follicular growth during controlled ovarian hyperstimulation with GnRH antagonists. *Hum Reprod*. 2003;18(12):2698–703.
277. Fanchin R, Cunha-Filho JS, Schonauer LM, et al. Coordination of early antral follicles by luteal estradiol administration provides a basis for alternative controlled ovarian hyperstimulation regimens. *Fertil Steril*. 2003;79(2):316–21.
278. Reynolds KA, Omurtag KR, Jimenez PT, et al. Cycle cancellation and pregnancy after luteal estradiol priming in women defined as poor responders: A systematic review and meta-analysis. *Hum Reprod*. 2013;28(11):2981–9.
279. Fanchin R, Castelo Branco A, et al. Premenstrual administration of gonadotropin-releasing hormone antagonist coordinates early antral follicle sizes and sets up the basis for an innovative concept of controlled ovarian hyperstimulation. *Fertil Steril*. 2004;81(6):1554–9.
280. Rombaerts L, Suikkari AM, MacLachlan V, et al. Recruitment of follicles by recombinant human follicle-stimulating hormone commencing in the luteal phase of the ovarian cycle. *Fertil Steril*. 1998;69(4):665–9.
281. Dragisic KG, Davis OK, Fasouliotis SJ, Rosenwaks Z. Use of a luteal estradiol patch and a gonadotropin-releasing hormone antagonist suppression protocol before gonadotropin stimulation for in vitro fertilization in poor responders. *Fertil Steril*. 2005;84(4):1023–6.
282. Frattarelli JL, Hill MJ, McWilliams GD, et al. A luteal estradiol protocol for expected poor-responders improves embryo number and quality. *Fertil Steril*. 2008;89(5):1118–22.
283. DiLuigi AJ, Engmann L, Schmidt DW, et al. A randomized trial of microdose leuprolide acetate protocol versus luteal phase ganirelix protocol in predicted poor responders. *Fertil Steril*. 2011;95(8):2531–3.
284. Weitzman VN, Engmann L, DiLuigi A, et al. Comparison of luteal estradiol patch and gonadotropin-releasing hormone antagonist suppression protocol before gonadotropin stimulation versus microdose gonadotropin-releasing hormone agonist protocol for patients with a history of poor in vitro fertilization outcomes. *Fertil Steril*. 2009;92(1):226–30.
285. Shastri SM, Barbieri E, Kligman I, et al. Stimulation of the young poor responder: Comparison of the luteal estradiol/gonadotropin-releasing hormone antagonist priming protocol versus Oral contraceptive microdose leuprolide. *Fertil Steril*. 2011;95(2):592–5.
286. Luteal phase estradiol versus luteal phase estradiol and antagonist protocol for controlled ovarian stimulation before in vitro fertilization in poor responders. Elassar A, Mann JS, Engmann L, Nulsen J, Benadiva C. *Fertil Steril*. 2011 Jan;95(1):324–6.
287. Letrozole and gonadotropins versus luteal estradiol and gonadotropin-releasing hormone antagonist protocol in women with a prior low response to ovarian stimulation. Elassar A, Engmann L, Nulsen J, Benadiva C. *Fertil Steril*. 2011 Jun;95(7):2330–4.
288. Cobo A, Garrido N, Crespo J, et al. Accumulation of oocytes: A new strategy for managing low-responder patients. *Reprod Biomed Online*. 2012;24(4):424–32.
289. Chatziparasidou A, Nijs M, Moisidou M, et al. Accumulation of oocytes and/or embryos by vitrification: A new strategy for managing poor responder patients undergoing pre implantation diagnosis. *F1000Research*. 2013;2:240.
290. Datta AK, Campbell S, Felix N, Nargund G. Accumulation of embryos over 3 natural modified IVF (ICSI) cycles followed by transfer to improve the outcome of poor responders. *Facts Views Vis Obgyn*. 2019;11(1):77–84.

THE POSEIDON STRATIFICATION OF “LOW-PROGNOSIS PATIENTS IN ART”

Management Strategies and Outcomes

Sandro C. Esteves, Hakan Yarali, Peter Humaidan, and Carlo Alvaggi

Introduction

The management of infertility patients with poor or suboptimal ovarian response to ovarian stimulation has been an area with unmet clinical needs. Besides the limited understanding of the underlying pathophysiology of poor or suboptimal ovarian response, there is also a wide variation in the definition of poor responders and overall disappointing outcomes when these patients undergo assisted reproductive technology (ART).

To further elaborate on these aspects, a group of experts, mainly clinicians specialized in reproductive medicine, created the POSEIDON group in 2015. POSEIDON is an acronym that stands for **P**atient-**O**riented **S**trategies **E**ncompassing **I**ndividualize**D** Oocyte Number. Since its creation, the group has worked on many projects, and it grew, having to date more than 100 members from various countries across the globe. The POSEIDON group is open to all interested in this field. Information about the POSEIDON group and how to join can be found on the POSEIDON website at www.grouposeidon.com.

The proposal of novel criteria to identify and classify patients with low prognosis in ART represents the most remarkable achievement of the POSEIDON group to date. The criteria were introduced in 2016 with the primary goal of underlining differences related to poor or suboptimal infertility treatment outcomes regarding oocyte quantity and quality and to possibly create more homogenous groups for clinical management and research [1, 2]. The new classification system is timely and clinically relevant because responses to gonadotropin stimulation and ART outcomes are highly variable, depending on individual patient factors. In particular, the POSEIDON criteria consider various features that affect treatment outcomes in patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI), including the number of oocytes retrieved after ovarian stimulation, the age-related embryo/blastocyst aneuploidy rate, and the ovarian “sensitivity” to exogenous gonadotropins [3, 4]. While the latter impacts the number of oocytes retrieved and might relate to specific genetic profiles, female age modulates the prognosis of patients with the same oocyte yield.

The POSEIDON criteria and rationale

The POSEIDON criteria classify patients with infertility undergoing ART into four groups of “low prognosis” based on female age and ovarian reserve markers (antral follicle count [AFC] and/or anti-Müllerian hormone [AMH]), also taking into consideration the number of oocytes retrieved after a standard ovarian stimulation, i.e. using gonadotropin starting doses of 150 IU or higher—if the patient had a previous ovarian stimulation cycle [1, 2].

As shown in Figure 53.1, groups 1 and 2 comprise patients with good ovarian reserve markers but who, unexpectedly, end up having poor (fewer than four) or suboptimal [4–9] oocytes retrieved

after a standard ovarian stimulation with gonadotropins. By contrast, groups 3 and 4 include patients with a low ovarian reserve and, as a result, an expected poor ovarian response to gonadotropin stimulation. Patients are further classified according to the age threshold of 35 years into young POSEIDON patients, i.e. groups 1 and 3, and older counterparts, i.e. groups 2 and 4. By contrast, patients with good ovarian reserve markers and normal response to ovarian stimulation (i.e. more than nine oocytes retrieved) can be classified as having a “normal” prognosis and are thus termed “non-POSEIDON” patients.

Female age is a critical pillar of the POSEIDON classification because it directly affects oocyte quality and embryo ploidy. In a 2019 study, analysing 1296 trophectoderm biopsies by next-generation sequencing from 436 infertile couples undergoing IVF/ICSI, we showed that the probability of having genetically normal embryos decreased with age in a non-linear manner [5] (Figure 53.2a). While the geometric mean of the yearly decrease was 13.6% overall, the probability of blastocyst euploidy followed an age-dependent binomial distribution, decreasing with every year of age—relative to the previous year—from 1.2% at age 29 to 2.0%, 3.5%, 6.7%, 9.8%, 13.6%, 17.9%, and 24.5% at ages 30, 32, 35, 37, 39, and 41, respectively ($p < 0.0001$) (Figure 53.2b). As shown in Figure 53.2a, the blastocyst euploidy probability sharply declined in patients older than 34 years. Notably, among patients aged 35 years and older, the chances of having a euploid blastocyst were below overall 50%. Consequently, these patients will likely require more oocytes and embryos to potentially overcome the adverse effect of aging on oocyte and embryo quality [5]. On this basis, 35 years was the age threshold adopted by the POSEIDON criteria to distinguish young and older low-prognosis patients [1, 2].

AFC and AMH serum levels are also included in the POSEIDON classification system because they are regarded as the best predictors of ovarian response and are widely used in routine clinical practice [6]. These markers predict poor response and retrieval of less than four or five oocytes after standard ovarian stimulation with good accuracy and acceptability [7]. The AFC and AMH thresholds proposed by the POSEIDON group were based on the published literature. Specifically, the AFC threshold of 5, incorporated into the POSEIDON criteria to distinguish patients with poor or adequate ovarian reserve, was based on 2-dimensional (2D) transvaginal sonography studies for AFC quantification [7]. Along these lines, the AMH cut-off value of 1.2 ng/mL was considered suitable for the same purpose, based on studies utilizing the manual enzyme-linked immunosorbent assay (ELISA) [7–10]. On this basis, patient classification using the AFC and/or AMH thresholds established by the POSEIDON criteria is method-specific. However, there is still no international standard for AMH; AMH values generated by different assays can be markedly different, and assay-specific interpretation is required. Since automated assays produce lower values than manual assays, patient classification will likely differ if the former is utilized.

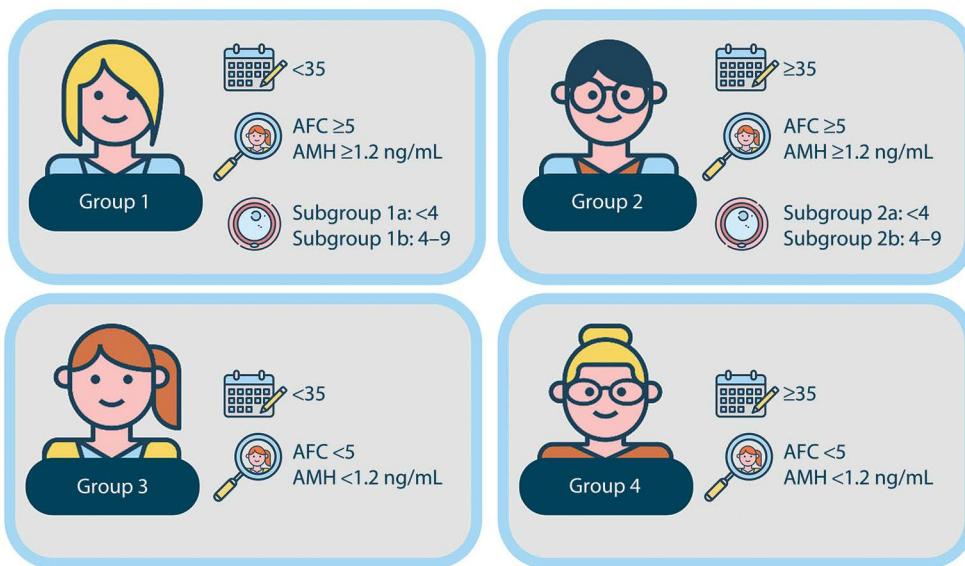


FIGURE 53.1 POSEIDON criteria. Four distinct groups of low-prognosis patients can be established based on quantitative and qualitative parameters, namely, (i) the age of the patient and its related embryo aneuploidy rate; (ii) ovarian biomarkers (antral follicle count [AFC] and/or anti-Müllerian hormone [AMH]), and (iii) the ovarian response in terms of oocyte number (if a previous cycle of conventional ovarian stimulation was carried out). Group 1: patients <35 years with sufficient pre-stimulation ovarian reserve parameters (AFC ≥ 5, AMH ≥ 1.2 ng/mL) and with an unexpected poor (fewer than four oocytes) or suboptimal (four to nine oocytes) ovarian response. This group is further divided into subgroup 1a, constituted by patients with fewer than four oocytes, and subgroup 1b, constituted by patients with four to nine oocytes retrieved after standard ovarian stimulation, who, at any age, have a lower live birth rate than age-matched normal responders. Group 2: patients ≥35 years with sufficient pre-stimulation ovarian reserve parameters (AFC ≥ 5, AMH ≥ 1.2 ng/mL) and with an unexpected poor or suboptimal ovarian response. This group is further divided into subgroup 2a, constituted by patients with fewer than four oocytes, and subgroup 2b, constituted by patients with four to nine oocytes retrieved after standard ovarian stimulation, who, at any age, have a lower live birth rate than age-matched normal responders. Group 3: patients <35 years with poor ovarian reserve pre-stimulation parameters (AFC < 5, AMH < 1.2 ng/mL). Group 4: patients ≥35 years with poor ovarian reserve pre-stimulation parameters (AFC < 5, AMH < 1.2 ng/mL). (Art drawing courtesy of Chloé Xilinas, Med. E.A., Rome, Italy. Reprinted from [47], copyright © 2021. This article is distributed under the Creative Commons Attribution License [CC BY].)

Accordingly, there is a need to adjust the cut-off value of 1.2 to 0.96 ng/mL if the AMH Elecsys assay is applied [11].

The POSEIDON criteria also consider the number of oocytes retrieved during ovarian stimulation because it is an independent predictor for live birth. This association is more pronounced when frozen-thawed cycles are considered. In a 2016 cohort study including 1099 consecutive women 18–40 years old undergoing their first IVF cycle and planning to undergo single embryo transfer (SET) in their fresh cycle, the ovarian response category was an independent predictive factor ($p < 0.001$) for cumulative live birth rate (LBR) [12]. The authors grouped patients according to the number of oocytes retrieved following a standard ovarian stimulation regimen with gonadotropins, namely, 1–3 (poor), 4–9 (suboptimal), 10–15 (normal), or >15 oocytes (high responders). After adjusting for female age, it was shown that although suboptimal responders had a better outcome than poor ovarian responders ($p = 0.002$), both groups had a significantly lower cumulative LBR per aspiration cycle than normal ($p = 0.02$) and high ovarian responders ($p < 0.001$). This and other studies substantiate the notion that lower oocyte yields are associated with lower delivery rates, particularly cumulative delivery rates per initiated or aspiration IVF/ICSI cycle. For this reason, and given the fact that the assessment of ovarian reserve using AFC and/or AMH cannot fully explain the individual response to ovarian stimulation, particularly in women with sufficient ovarian

parameters [13], oocyte thresholds, defined as poor [1–4] or suboptimal [4–9], obtained after a standard ovarian stimulation with gonadotropins, are also included in the POSEIDON classification. These ovarian response categories are associated, at any given age, with significantly lower cumulative delivery rates than that obtained in normal responders [12].

Overall, POSEIDON patients can be summarized as illustrated in Figure 53.3. In groups 1 and 3, patients are young and, consequently, the risk of embryo aneuploidy is relatively low. By contrast, patients of groups 2 and 4 are older, and, as a result, the risk of embryo aneuploidy is increased. The bottom line is that the number of embryos generated will likely be low in all categories, thus affecting the cumulative delivery rate per initiated or aspirated IVF/ICSI cycle. POSEIDON patients are regarded as having “low prognosis” in ART because they are at a higher risk of failing to achieve a live birth after IVF/ICSI treatment than normal responders with an adequate ovarian reserve. However, it has been suggested that their prognosis varies according to the group (i.e. groups 1 to 4, based on age, ovarian reserve, and the number of oocytes retrieved) [3].

Under the POSEIDON criteria, the cumulative delivery rate per initiated/aspiration IVF/ICSI cycle is proposed to be the crucial endpoint that sets the prognosis of patients apart. The International Committee for Monitoring Assisted Reproductive Technologies (ICMART) defines this metric as “the

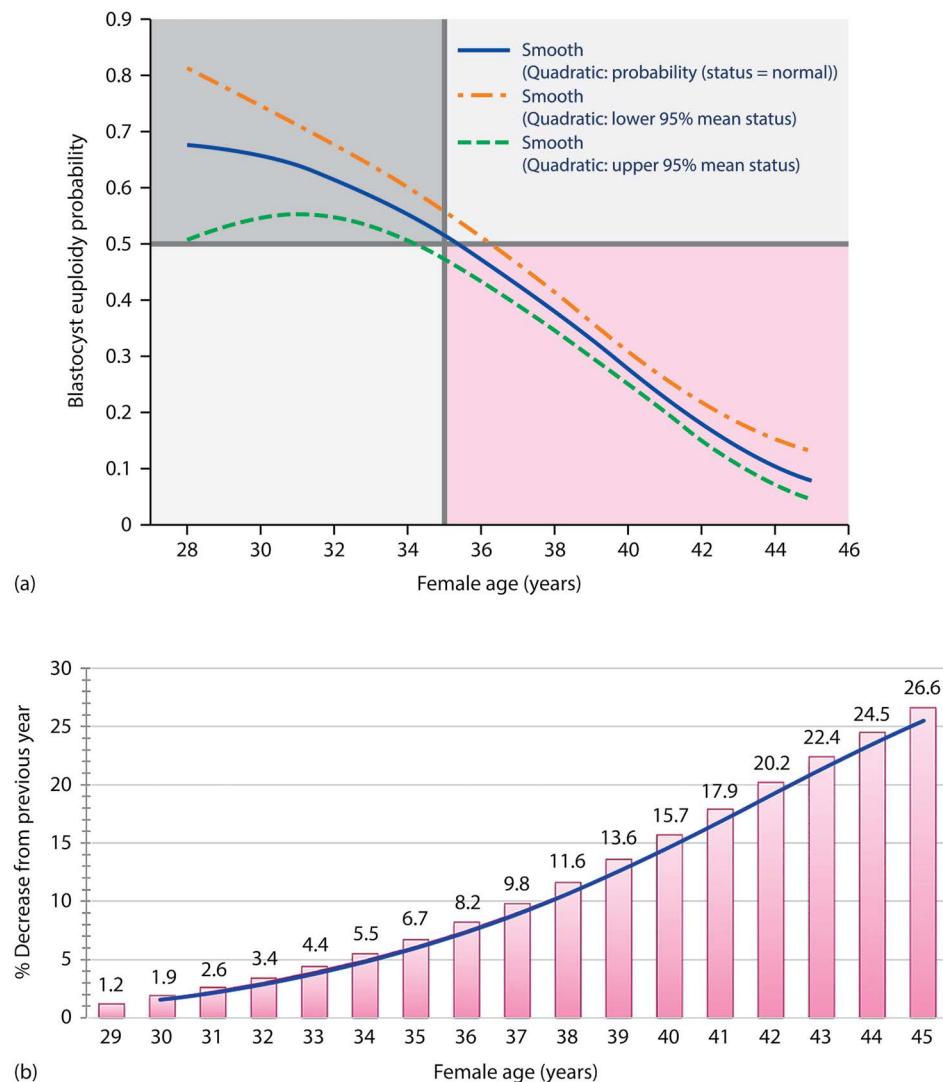


FIGURE 53.2 Impact of female age on blastocyst euploidy. Logistic regression analysis of 1220 trophectoderm biopsies from 436 infertile couples undergoing ICSI and pre-implantation genetic testing for aneuploidy (PGT-A) by next-generation sequencing. The dependent and independent variables were embryo genetic status (euploid/aneuploid) and female age, respectively. (a) The plot depicts the fitted probabilities (with 95% confidence intervals; dotted lines) of blastocyst euploidy as a function of female age. (b) The graph shows the yearly percent decrease in the probability of blastocyst euploidy, which increases progressively with every year of female age. The percentage decrease in blastocyst euploidy probability from year (t) to year (t+1) was defined as the ratio $p(t+1)/p(t) \times 100$. (Reprinted with permission of Edizioni Minerva Medica from [5].)

number of deliveries with at least one live birth resulting from one initiated or aspirated ART cycle, including all cycles in which fresh and/or frozen embryos are transferred until one delivery with a live birth occurs or until all embryos are used, whichever occurs first, expressed per 100 cycles (initiated or aspirated)¹⁴. The cumulative delivery rate has been regarded as the most appropriate to report ART success^[15, 16] and was included in the ESHRE 2019 guideline on ovarian stimulation for IVF/ICSI^[17] as a critical efficacy outcome. Furthermore, it is considered the most meaningful outcome from the patients' perspective because it adequately reflects the prognosis of achieving a live birth after one initiated/aspirated ART cycle^[18].

Clinical validation

Although the POSEIDON stratification makes sense from a theoretical point of view, there was a need to validate its clinical utility. Firstly, it was essential to determine how often POSEIDON patients are seen in the fertility clinic. Secondly, it was pivotal to validate the AFC and AMH cut-off points proposed by the POSEIDON group and determine whether AFC and AMH could be used interchangeably for patient classification. Lastly, it was necessary to confirm whether POSEIDON patients had indeed “low prognosis” compared to non-POSEIDON patients and determine whether the prognosis differed among POSEIDON groups.

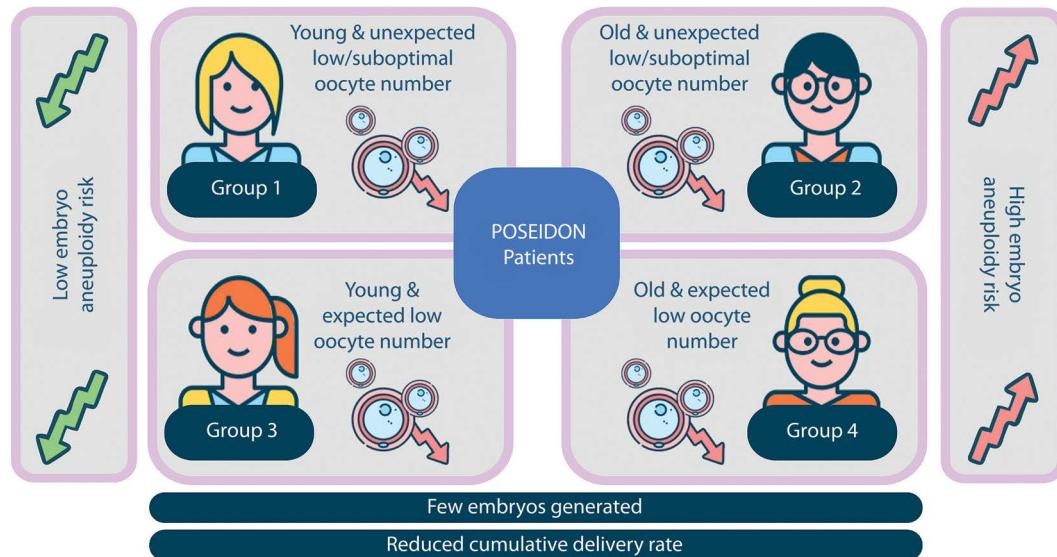


FIGURE 53.3 Snapshot of POSEIDON's criteria clinical significance. Groups 1 and 3 patients are young, and, consequently, their risk of embryo aneuploidy is relatively low. Patients of groups 2 and 4 have advanced maternal age, and, as a result, their risk of embryo aneuploidy is increased. The bottom line is that the number of embryos generated will likely be low in all categories, thus affecting the cumulative delivery rate per initiated or aspirated IVF/ICSI cycle.

To answer these questions, the POSEIDON group conducted three studies using real-world evidence (RWE), discussed in the following sections.

How often are POSEIDON patients encountered in routine practice?

The prevalence of POSEIDON patients in routine clinical practice was estimated in a multicentre study including 13,146 consecutive infertile women aged 22–46 years and treated in the three fertility clinics in Brazil, Turkey, and Vietnam [19]. Eligible patients were those who had their first IVF/ICSI cycle in each centre and used standard ovarian stimulation using exogenous gonadotropins (i.e. ≥150 IU daily doses of exogenous gonadotropin). AFC was the biomarker used for patient classification.

In this report, POSEIDON patients accounted for 43% (95% confidence interval [CI]: 42.0–43.7%) of the overall studied population, but the prevalence varied across study centres (range: 38.6–55.7%). The prevalence rates by POSEIDON groups were 44.2% (group 1; 95% CI: 42.6–45.9%), 36.1% (group 2; 95% CI: 34.6–37.7%), 5.2% (group 3; 95% CI: 4.5–6.0%), and 14.4% (group 4; 95% CI: 13.3–15.6%), thus indicating that most patients had an unexpected poor or suboptimal response to ovarian stimulation despite having sufficient pre-stimulation ovarian biomarkers (Figure 53.4).

Female age, body mass index (BMI), AFC, and presence of a female infertility factor were significant predictors of the POSEIDON condition. In general, POSEIDON patients were older (34.0 years, interquartile range [IQR] 31.0–38.0 vs 31.0 years, IQR 28.0–35.0; $p < 0.001$), had a higher BMI (22.0, IQR: 20.0–24.5 vs 21.3, IQR 19.8–23.7; $p < 0.001$), had lower ovarian reserve markers (AFC: 8, IQR 5–12 vs 17, IQR 13–23, $p < 0.001$; AMH: 1.5 ng/mL, IQR 0.9–3.0 vs 4.9, IQR 3.0–7.8, $p < 0.001$), and had a higher frequency of female factor (69.3% vs 63.2; $p < 0.001$) as the primary treatment indication than non-POSEIDON patients. Moreover, POSEIDON patients required larger doses of gonadotropin for

ovarian stimulation (2700 IU, IQR: 1100–5100 vs 2300 IU, IQR 1050–4464; $p < 0.001$) despite achieving a 2.5 times lower number of retrieved oocytes (6, IQR 4–8 vs 15, IQR 12–19; $p < 0.001$) than non-POSEIDON patients (i.e. patients with ovarian markers above the thresholds established by the POSEIDON criteria and who had more than nine oocytes retrieved after a standard ovarian stimulation with exogenous gonadotropins) [19].

Collectively, this RWE study was the first to report global prevalence estimates of POSEIDON patients. It showed that POSEIDON patients are commonly seen in routine practice. The main traits of these patients (vs normal responders) include advanced female age, decreased ovarian reserve, increased BMI, and the presence of a female infertility factor. These findings may help decision-makers and practitioners implement measures to mitigate the risk of low prognosis and optimize reproductive planning. For instance, awareness campaigns could stress the importance of female age and ovarian reserve on reproductive success, lifestyle changes, early diagnosis, and the potential role of individualized treatment strategies to improve treatment success.

Which biomarker to use for patient classification: AFC, AMH, or both?

Under the POSEIDON system, ovarian biomarkers, specifically AFC and AMH, must be used for patient classification [1, 2]. However, the criteria do not make explicit recommendations regarding which marker to use or whether results obtained from both markers should be combined to make a judgment. With this in mind, we sought to determine the agreement between AFC and AMH within this context. Secondly, we assessed whether the AFC and AMH thresholds put forth by the POSEIDON group were accurate enough to predict a poor response to standard ovarian stimulation.

For this, we conducted an RWE multicentre agreement study including 9484 consecutive patients between 22 and 46 years

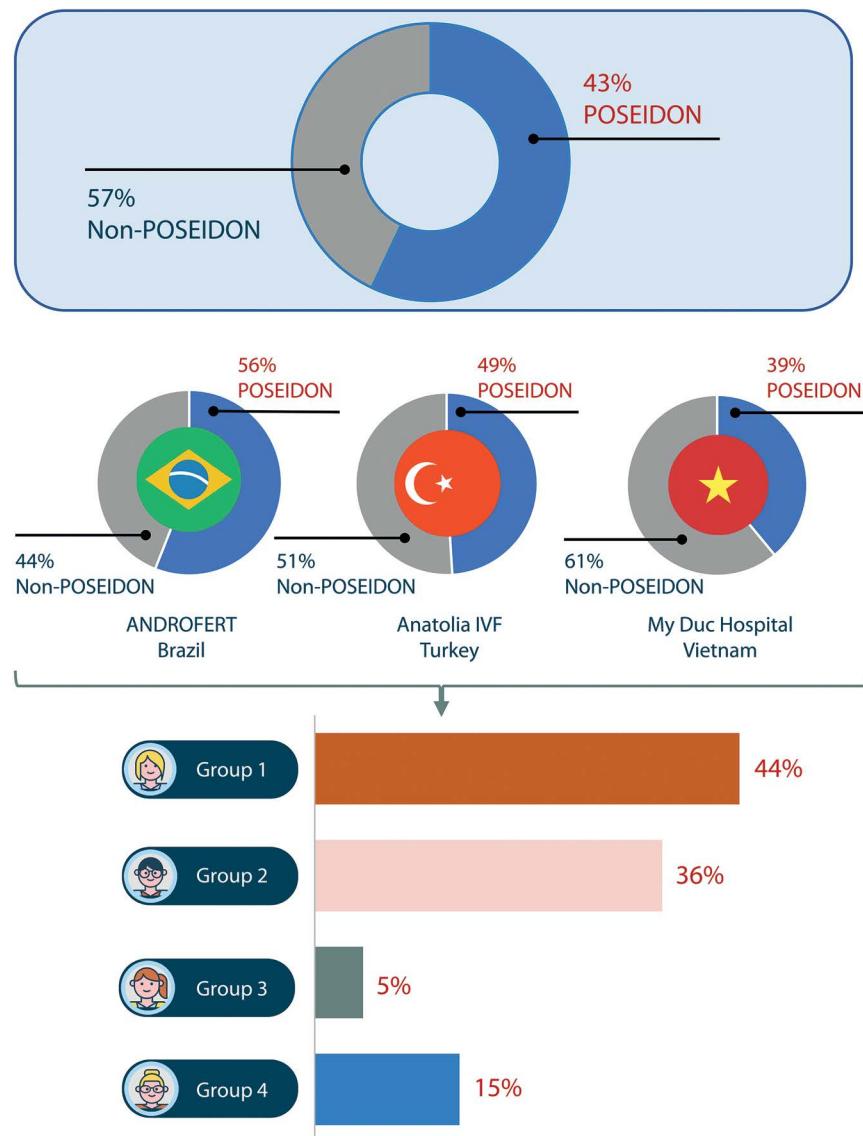


FIGURE 53.4 Prevalence of low-prognosis patients under the POSEIDON criteria. Data from a multicentre population-based study of 13,146 patients showed POSEIDON patients represented 42.9% (95% confidence interval [CI] 42.0–43.7) of the studied population, and the prevalence rates varied across study centres. (Androfert Brazil: 55.7%, 95% CI 52.7–58.6; Anatolia IVF Turkey: 49.1%, 95% CI 47.4–50.7; My Duc Hospital Vietnam: 38.6%, 95% CI 36.7–39.7). The overall prevalence rates by POSEIDON groups were 44.2% (group 1; 95% CI 42.6–45.9), 36.1% (group 2; 95% CI 34.6–37.7), 5.2% (group 3; 95% CI 4.5–6.0), and 14.4% (group 4; 95% CI: 13.3–15.6). (Adapted from [19], Copyright © 2021. This article is distributed under the Creative Commons Attribution License [CC BY].)

old in their first IVF/ICSI cycle of standard ovarian stimulation with exogenous gonadotropins whose baseline ovarian reserve had been assessed by both AFC and AMH [20]. The AFC was determined in the early follicular phase using 2D transvaginal ultrasonography, whereas AMH values were obtained by the modified Beckman Coulter generation II ELISA. Our results indicated a strong agreement ($\kappa = 0.802$; 95% CI: 0.792–0.811) between AFC and AMH levels in classifying patients according to POSEIDON criteria. Three out of every four patients were classified under the same group using both biomarkers in practical terms.

The disagreement rate, that is, patients classified under a different group due to discordant biomarker results, was similar (about 26%) when either AFC or AMH was used as the primary

biomarker criterion (Figure 53.5). However, among discrepant patients, a “true” poor ovarian response (i.e. fewer than four oocytes retrieved) was more frequently observed (44.9% vs 27.1%) when AFC was used as the primary biomarker for POSEIDON classification, possibly because the AFC threshold of 5 is more restrictive and conservative than the corresponding AMH of 1.2 ng/mL. Logistic regression analysis examining possible causal associations of patient and treatment covariates within the group of patients with discordant results indicated that the discrepancy was primarily associated with technical limitations of biomarkers’ measuring methods, particularly AMH. Nevertheless, virtually all patients with conflicting biomarker results remained within the broad category of “low prognosis”; this means that the risk of having a patient initially classified as belonging to one of

POSEIDON classes by AFC	N (%)	POSEIDON classes by AMH							Total
		1a	1b	2a	2b	3	4	5	
1a	86 (55.1)	0 (0.0)	0 (0.0)	0 (0.0)	70 (44.9)	0 (0.0)	0 (0.0)	0 (1.6)	156
	0 (0.0)	1093 (83.1)	0 (0.0)	0 (0.0)	223 (16.9)	0 (0.0)	0 (0.0)	0 (13.9)	1316
	0 (0.0)	0 (0.0)	118 (44.5)	0 (0.0)	0 (0.0)	147 (55.5)	0 (0.0)	0 (2.8)	265
	0 (0.0)	0 (0.0)	0 (0.0)	906 (71.5)	0 (0.0)	361 (28.5)	0 (0.0)	0 (13.4)	1267
	29 (11.2)	41 (15.8)	0 (0.0)	0 (0.0)	189 (73.0)	0 (0.0)	0 (0.0)	0 (2.7)	259
	0 (0.0)	0 (0.0)	72 (11.0)	83 (12.7)	0 (0.0)	496 (75.7)	4 (0.6)	0 (6.9)	655
	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	76 (1.4)	66 (1.2)	5424 (97.4)	5566 (58.7)	5566
	115 (1.2)	1134 (11.9)	190 (2.1)	989 (10.4)	558 (5.9)	1070 (11.3)	5428 (57.2)	9484	9484

FIGURE 53.5 Biomarkers' agreement in POSEIDON criteria. Contingency table showing the frequency distribution of patients by group, classified using antral follicle count (AFC) and anti-Müllerian Hormone (AMH) levels. Values in bold and highlighted (yellow background) represent the agreement frequencies between the two biomarkers falling into the same POSEIDON group. (Reprinted with permission of Oxford University Press from [20].)

the POSEIDON groups crossing the line and deemed as a non-POSEIDON patient—if the alternate biomarker had been used—was negligible [20].

Another finding from this study was that the optimal AFC and AMH thresholds to predict the retrieval of fewer than four oocytes were 5 and 1.27 ng/mL, respectively, with sensitivities of 0.61 and 0.66, specificities of 0.81 and 0.72, and area under the receiver operating characteristics curve (AUC) of 0.791 and 0.751, respectively, and thus like the values established by the POSEIDON criteria [20]. Furthermore, AFC and AMH thresholds were provided for the first time to identify suboptimal responders, i.e. patients who end up with an oocyte yield between four and nine after standard ovarian stimulation. An AFC of 12 and an AMH value of 2.95 ng/mL were the optimal thresholds for a “suboptimal” oocyte retrieval after traditional ovarian stimulation, defined as the retrieval of four to nine oocytes, with sensitivities of 0.74 and 0.69, specificities of 0.76 and 0.66, and AUCs of 0.81 and 0.80, respectively (Figure 53.6).

Collectively, the study just discussed indicated a strong agreement between the AFC and the AMH levels in classifying POSEIDON patients. Although one in four women will have discordant values when both biomarkers are used, patient classification disagreement rates do not seem to be materially affected by whether AFC or AMH is used as the biomarker primary criterion. Moreover, no evident superiority of one marker over the other was noted concerning predicting the number of oocytes retrieved as indicated by equivalent ROC curves. Our data support the notion that AFC and AMH may be used interchangeably for patient classification under the POSEIDON criteria. Combining both biomarkers for that purpose brings little practical value. On this basis, clinicians should adopt the biomarker that best reflects their clinical setting.

Is the prognosis of POSEIDON patients low?

To answer this question, we compared the cumulative delivery rates after one aspirated cycle among POSEIDON groups using a control group of normal responders as the reference population.

This RWE multicentre study included 9073 consecutive infertile women 22 and 42 years old in their first IVF/ICSI cycle of standard ovarian stimulation, in whom fresh and/or frozen embryos were transferred until a live birth delivery or until all embryos were used [21].

The survival plots showed that the cumulative delivery rates were significantly lower in POSEIDON patients than in non-POSEIDON patients (33.7% vs 50.6%, $p < 0.001$) (Figure 53.7). Among POSEIDON groups, cumulative delivery rates were higher in the younger population (group 1a: 27.8%; group 1b: 47.8%; group 3: 29.4%) than in the older population (group 2a: 14.0%; group 2b: 30.5%; group 4: 12.5%). Within the non-POSEIDON group, the cumulative delivery rate was lower in older (≥ 35 years) than in younger (< 35 years) patients (56.4% vs 34.8%, $p = 0.004$). Nevertheless, the cumulative delivery rate in older (≥ 35 years) non-POSEIDON patients was higher than that of POSEIDON suboptimal responders of a similar age stratum (group 2b: 30.5%; $p = 0.03$), indicating that oocyte yield modulated the cumulative delivery rates [21].

Along these lines, a logistic regression analysis showed that POSEIDON grouping, number of embryos obtained, number of embryo transfers per patient, number of oocytes retrieved, female age, infertility duration, and BMI were relevant predictors for the cumulative delivery rate ($p < 0.001$). Interestingly, within the group of unexpected suboptimal (four to nine oocytes) and poor (fewer than four oocytes) responders (POSEIDON groups 1 and 2), the cumulative delivery rate was twice as high in suboptimal responders as in poor responders (subgroups 1b and 1a: 47.8% vs 27.8%, $p = 0.0004$; subgroups 2b and 2a: 30.5% vs 14.0%, $p = 0.004$) (Figure 53.8), thus confirming the role of oocyte number on cumulative delivery rates [21].

Indeed, our findings confirmed that the number of oocytes retrieved translated into more embryos available for transfer. The frequency of suboptimal responders (four to nine oocytes; subgroups 1b and 2b) with supernumerary vitrified embryos was four times higher (14% vs 3.4%) than that observed in expected poor responders (groups 3 and 4) and ~17 times higher (14% vs 0.8%)

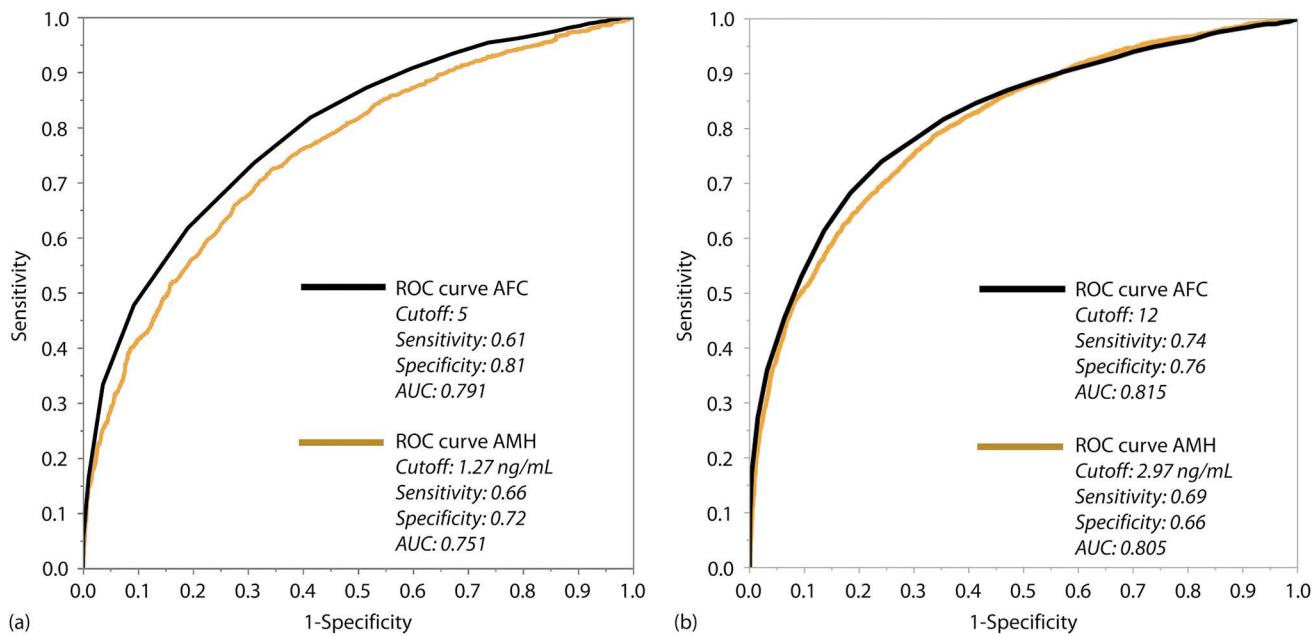


FIGURE 53.6 Validation of POSEIDON criteria biomarkers' thresholds. The figure shows the age-adjusted ROC curves of AFC and AMH to predict low (fewer than four) and suboptimal (four to nine) oocyte yields. (a) For low oocyte yield, the optimal AFC cut-off value was 5, with a sensitivity of 0.61, a specificity of 0.81, positive and negative predictive values of 64.1% and 79.4%, and an AUC of 0.791. Moreover, the optimal AMH cut-off value was 1.27 ng/mL, with a sensitivity of 0.66, a specificity of 0.72, positive and negative predictive values of 56.7% and 79.4%, and an AUC of 0.751. For suboptimal oocyte yield, the optimal AFC cut-off value was 12, with a sensitivity of 0.74, a specificity of 0.76, and an AUC of 0.81. Accordingly, the optimal AMH cut-off was 2.97 ng/mL, with a sensitivity of 0.69, a specificity of 0.66, and an AUC of 0.80. (Reprinted with permission of Oxford University Press from [20].)

than in unexpected poor responders (fewer than four oocytes retrieved; subgroups 1a and 2a). By contrast, 37% of non-POSEIDON patients had supernumerary vitrified embryos. As a result, more suboptimal responders and non-POSEIDON patients (vs poor responders) received a subsequent embryo transfer after the first failed cycle, thereby increasing the cumulative delivery rate in these patients [21]. Naturally, the positive impact of supernumerary embryos on the cumulative delivery rate depends on efficient embryo vitrification protocols [22].

Collectively, this study showed that the cumulative delivery rates of POSEIDON patients are, on average, 50% lower than normal responders and varied across POSEIDON groups. The lower cumulative delivery rates in POSEIDON patients (vs non-POSEIDON patients) are not surprising, as fewer oocytes are retrieved, and fewer embryos are obtained in these patients after one stimulation cycle, limiting the number of embryos available for transfer. Nevertheless, the impact of POSEIDON classification on the cumulative delivery rate is attenuated in patients with increased oocyte yields, i.e. suboptimal responders (vs poor responders), primarily due to the availability of supernumerary vitrified embryos available for subsequent transfers—in case the first failed. Moreover, the impact of POSEIDON classification on cumulative delivery rates is reduced in younger patients by the well-known protective age-related effect exerted on oocyte/embryo quality in these women. The preceding findings substantiate the validity of the POSEIDON criteria in identifying relevant subpopulations of patients with low prognosis in IVF/ICSI treatment.

POSEIDON metric of success and “ART” calculator

Given the importance of oocyte number and female age for cumulative delivery rates, the POSEIDON group proposed a new objective metric to measure success in ART, namely, “[t]he ability to retrieve the number of oocytes needed to obtain at least one euploid blastocyst for transfer in each patient” [1, 2]. Embryo euploidy was chosen as the metric endpoint because (i) it is primarily determined by female age and (ii) implantation and live birth rates are relatively constant (~50%–60%) across all age groups after the transfer of euploid embryos [23]. This means that the availability of euploid embryos helps offset the adverse effect of female age on ART reproductive success and provides the couple with a decent probability of achieving a live birth delivery.

Data from our 2019 trophectoderm biopsy study mentioned previously showed that the frequency of patients with at least one euploid blastocyst is higher in younger patient groups, which is not surprising [5]. However, this study also showed that the higher the number of embryos obtained, the higher the frequency of women with at least one euploid embryo, irrespective of age (Figure 53.9). Therefore, a plausible solution to improve reproductive success, especially for patients of advanced age, would be to increase the number of metaphase II oocytes, leading to more embryos, which would increase the chances of at least one being euploid. Here is an important question: how many oocytes does a specific patient need?

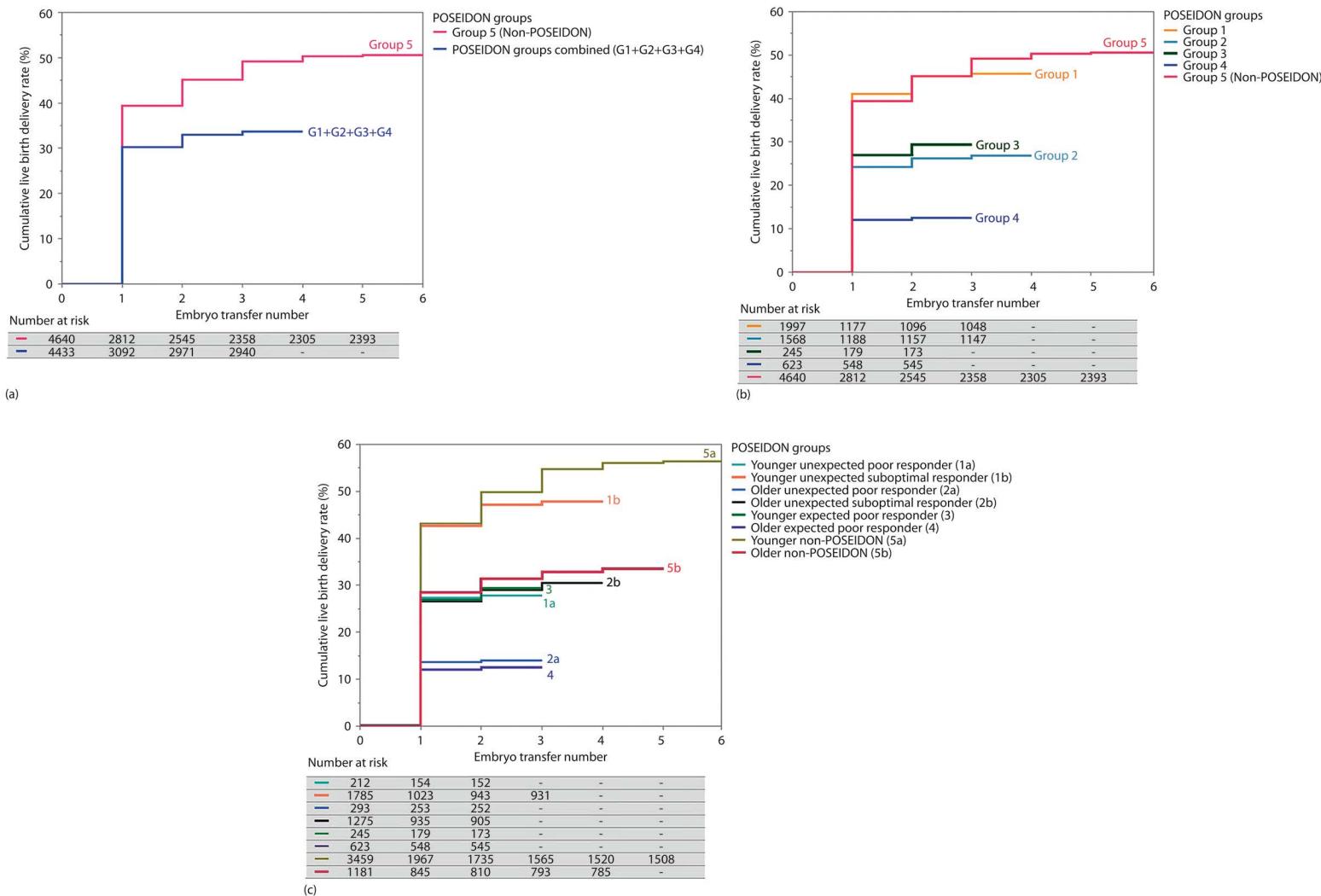


FIGURE 53.7 Survival plots for cumulative delivery in POSEIDON patients. Patients were stratified according to the POSEIDON criteria. Time-to-event plots were generated by (a) all POSEIDON patients—combined into a single group—and the control group of non-POSEIDON patients, (b) the four POSEIDON groups and the control group of non-POSEIDON patients, and (c) the four POSEIDON groups, in which groups 1 and 2 were further stratified in subgroups “a” and “b,” and non-POSEIDON patients further stratified by age using the 35-year threshold. Cumulative delivery rate survival functions were calculated using the nonparametric Kaplan–Meier method and non-censored values. The “time” response was the order of embryo transfers (ETs); the patient was the observational unit, and live birth delivery was the event. The survival tables detail the number of patients who failed to achieve a live birth delivery (number at risk). The tables are sectioned (columns) by each ET from one aspirated IVF/ICSI cycle (see ET order, 1, 2, 3 ... on the “x” axis of correspondent survival plots). Each group occupies its own row in the tables. The start of the tables (left column) indicates the number of patients who commenced treatment and had an oocyte pickup. The lines in each plot represent the cumulative proportion of patients achieving a live-born from the start of treatment until the “time” response. (Reprinted from [21]. This article is distributed under the Creative Commons Attribution License [CC BY].)

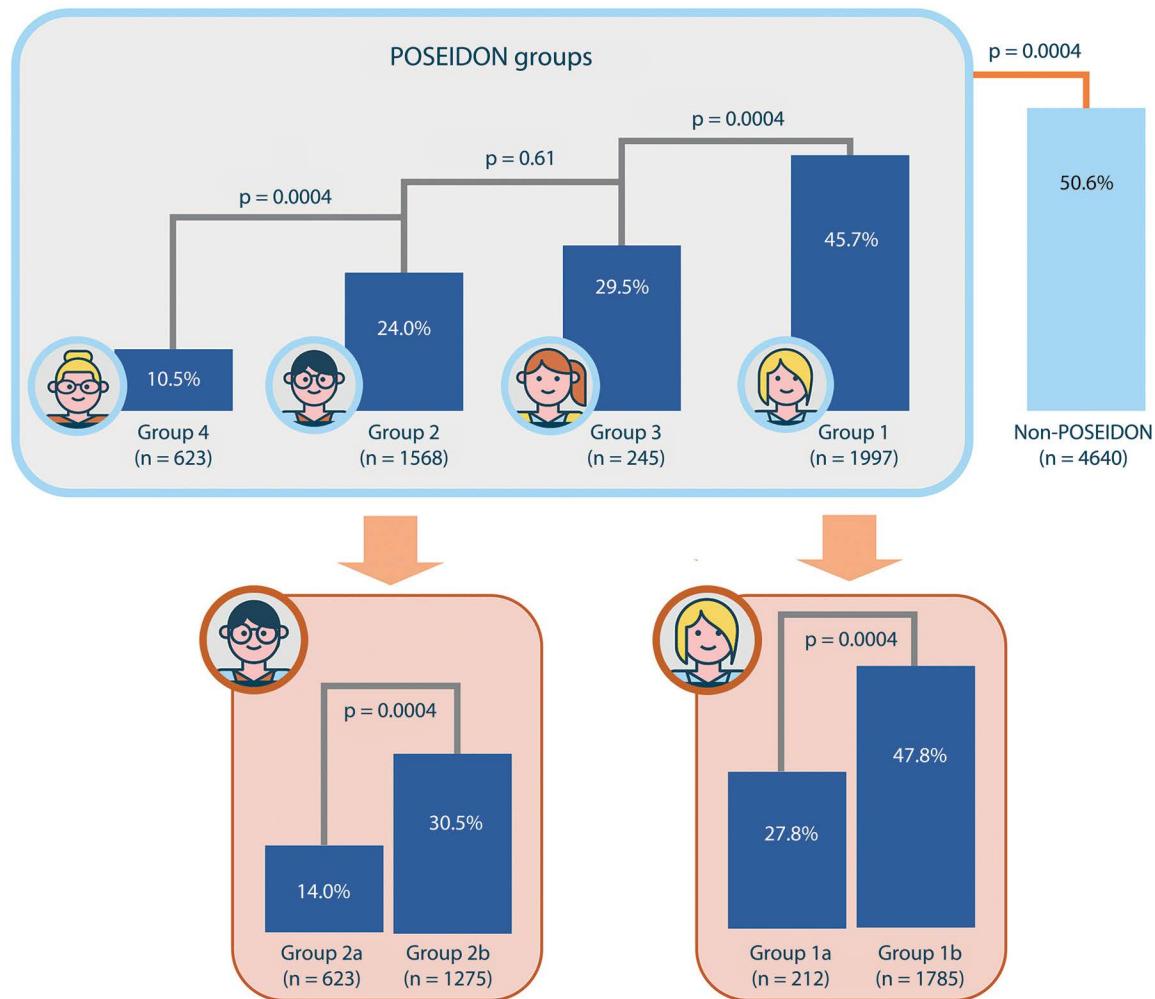


FIGURE 53.8 Cumulative delivery rates in POSEIDON patients. The prognosis of POSEIDON patients undergoing assisted reproductive technology, defined by the probability of achieving a delivery after the transfer of one or more embryos obtained from one aspiration IVF/ICSI cycle, is 50% lower than non-POSEIDON patients (adequate ovarian reserve biomarkers and more than nine oocytes retrieved) and varied across POSEIDON groups, primarily due to age and number of oocytes retrieved. The cumulative delivery rates are higher in younger POSEIDON patients (groups 1 and 3) than older counterparts (groups 2 and 4) and twice as high in suboptimal responders (four to nine oocytes retrieved: subgroups 1b and 2b) as in poor responders (fewer than four oocytes retrieved: subgroups 1a and 2a). Cumulative delivery rate was defined as the number of deliveries with one or more live births resulting from one aspiration IVF/ICSI cycle, including all cycles in which fresh and/or frozen embryos are transferred until one delivery with a live birth occurs or until all embryos are used, whichever occurs first. (Adapted from [21]. This article is distributed under the Creative Commons Attribution License [CC BY].)

To answer this question, the POSEIDON group developed a predictive model, called the “ART calculator,” using the clinical database of one of its members [24]. A generalized logistic regression analysis was used to select the variables significantly impacting the probability of embryo euploidy. Among 26 variables, including the age of the couple, ovarian biomarkers, infertility aetiology and duration, male factors (i.e. aetiology, semen parameters), treatment variables (i.e. stimulation regimen, type and dose of gonadotropins), and laboratory variables (e.g. number of oocytes, number of MII oocytes, sperm source for IVF/ICSI, fertilized oocytes [2PN], number of blastocysts, number of euploid blastocysts, and blastocyst euploidy rates), the model selected three relevant variables, namely, female age, type of

sperm used for ICSI, and MII oocytes. The fitted model provided an equation to estimate the individualized probability of blastocyst euploidy per MII oocyte as a function of female age and type of sperm used for ICSI. As shown in Figure 53.10, the likelihood of an MII oocyte turning into a euploid blastocyst decreases progressively with female age. The effect is negatively modulated by using testicular sperm from males with non-obstructive azoospermia (NOA).

Based on the model equation and other mathematical functions, an algorithm was developed to estimate the minimum number of MII oocytes to obtain at least one euploid blastocyst, with the correspondent 95% confident interval. The algorithm included a function, allowing users to set the probability

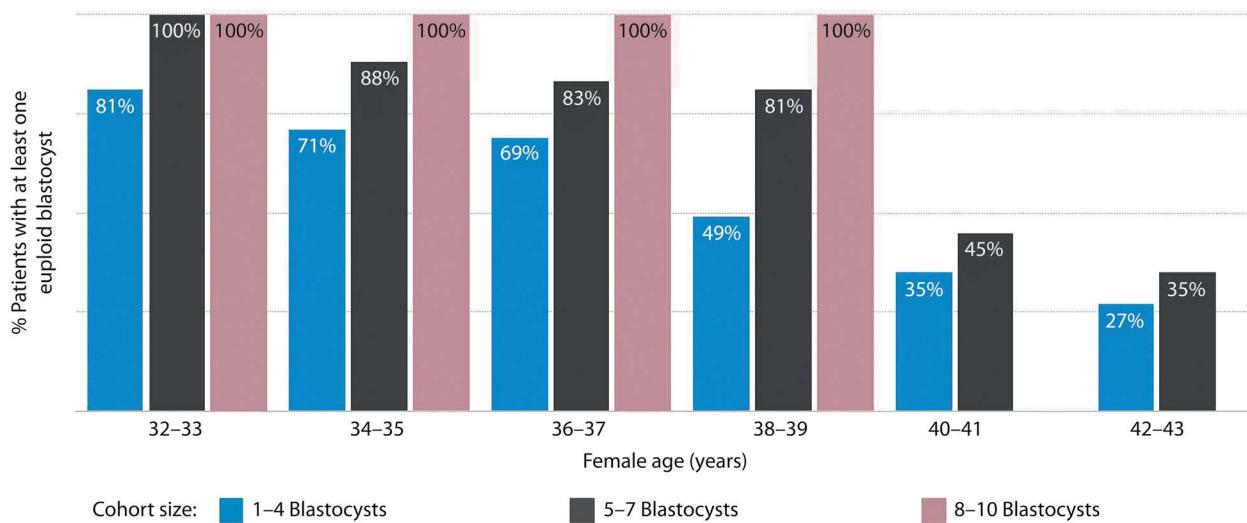


FIGURE 53.9 Frequency of patients with at least one euploid blastocyst stratified by age and embryo cohort size. Analysis of 1220 trophectoderm biopsies from 436 infertile couples undergoing ICSI and pre-implantation genetic testing for aneuploidy (PGT-A) by next-generation sequencing. The mean frequency of patients with at least one euploid blastocyst was lower in the small cohort size (one to four blastocysts) across all age groups than in the larger cohorts (five to seven and eight to ten blastocysts) ($P < 0.001$). (Reprinted with permission of Edizioni Minerva Medica from [5].)

of success for the estimations, for example, a 70%, 80%, or 90% certainty that at least one euploid embryo will be obtained if the calculated oocyte number is reached [24].

Essentially, the ART calculator makes two types of predictions. Pre-treatment, it estimates the minimum number of MII oocytes needed to achieve at least one euploid blastocyst for transfer in couples with infertility undergoing ART. To obtain an estimation, all clinicians must do is input the patient's age, the type of sperm used for IVF/ICSI, and set the probability of success for the estimation. The probability of success is denoted by π , and its complement, $1-\pi$, is the risk, i.e. the probability of having no euploid blastocyst despite achieving the minimum number of MII oocytes estimated [24]. Subsequently, with a single click, the calculator estimates the minimum number of MII oocytes needed to obtain at least one euploid blastocyst, with the correspondent range.

Post-treatment, if the number of oocytes—as estimated by the ART calculator—is not achieved, the application provides a revised estimation of the probability of achieving at least one euploid embryo with the number of MII oocytes ultimately obtained (Figure 53.11). The ART calculator can be found online at <http://www.members.groupposeidon.com/Calculator/>.

A 2020 study reported the ART calculator's validation results using data of 1464 IVF/ICSI patients from three fertility clinics in Italy, Turkey, and Brazil [25]. The validation study showed a strong correlation between the fittings of the original calculator and those obtained from the validation data set. The fittings were sufficiently close for both the estimated probabilities of blastocyst euploid per MII oocyte ($r = 0.91$) and the minimum number of MII oocytes required to obtain at least one euploid blastocyst ($r = 0.88$) [25]. These findings indicate that the algorithms obtained from the original ART calculator and validation data sets were similar and provided nearly identical outputs in estimating the oocyte number needed to get at least one euploid embryo. In the validation study, the ART calculator's positive predictive

value (i.e. frequency of patients with at least one euploid blastocyst among those who achieved the estimated minimum number of MII oocytes) was optimal (84.8%, 87.5%, and 90.0% for 70%, 80%, and 90% predicted probabilities of success, respectively) [25]. Therefore, in real-life settings, most patients who achieved the number of MII oocytes predicted by the ART calculator had at least one euploid blastocyst in their embryo cohort. The scientific validation of the ART calculator suggests that this application could be used in clinical practice for counselling and treatment planning [3].

Follicle-to-oocyte index (FOI)

The ratio between the number of oocytes retrieved at the ovum pickup and the total number of antral follicles at the start of ovarian stimulation (follicle-to-oocyte index [FOI]) has been proposed by the POSEIDON group as a novel metric to assess the effectiveness of ovarian stimulation [13] (Figure 53.12).

The FOI is suggested to reflect the dynamic nature of follicular growth in response to exogenous gonadotropins. It may be particularly informative in patients with unexpected suboptimal or poor responses to ovarian stimulation (i.e. POSEIDON groups 1 and 2), characterized by an unexpected poor (fewer than four) or suboptimal (four to nine) oocytes retrieved by use of standard age- and BMI-matched doses of exogenous gonadotropins despite sufficient pre-stimulation ovarian parameters [13]. In a 2020 observational study by Chen et al., including 32,128 fresh IVF cycles, the lowest FOI values were noted in patients of groups 2 (mean: 62%; 95% CI 59–64) and 1 (mean: 69%; 95% CI 67–71). By contrast, patients of groups 3 and 4, as well as non-POSEIDON patients, had FOI values of 80% or greater ($p < 0.001$) [27].

Accordingly, low FOI values might indicate a hypo-response to gonadotropin stimulation, implying that only a fraction of available antral follicles was adequately recruited. As a result, only a limited number of oocytes was retrieved [13, 27]. This

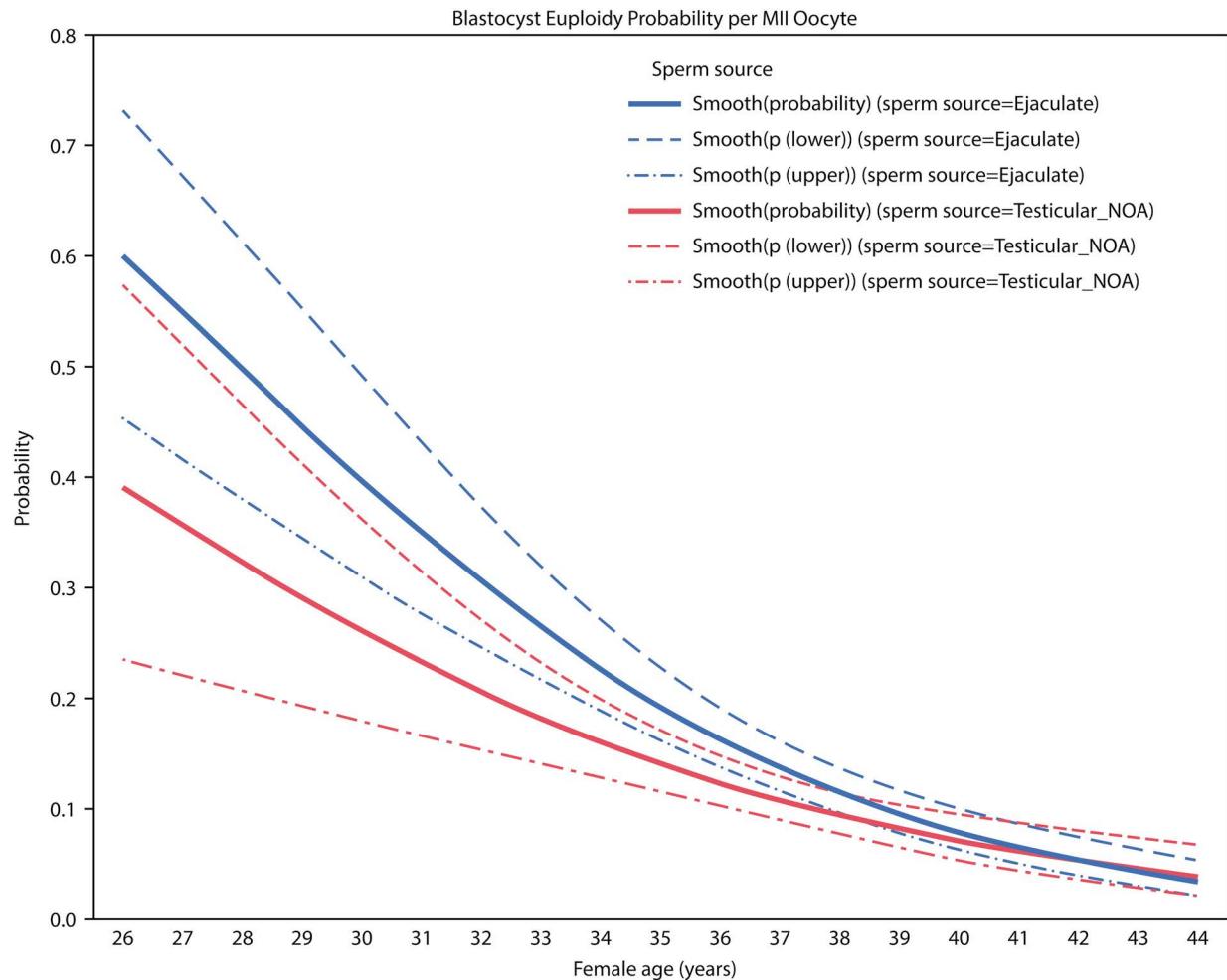


FIGURE 53.10 Blastocyst euploidy probability per metaphase II (MII) oocyte. The plots show the probability of a metaphase II (MII) oocyte turning into a euploid blastocyst as a function of female age. The estimated probabilities (solid curves) and their 95% confidence interval (dotted curves) are presented according to sperm source to be used for IVF/ICSI, namely, ejaculated sperm (blue) and testicular sperm extracted from patients with non-obstructive azoospermia (NOA) (red). The relations are non-linear and characterized by a differential modulatory effect of sperm source across age. The effect size of female age on blastocyst euploidy probability per MII oocyte from the year (t) to year ($t+1$) was defined as the ratio $p(t+1)/p(t) \times 100$. There was a significant decrease ($p < 0.001$) in the probability of an MII oocyte becoming a euploid blastocyst with aging. (Reprinted from [24], Copyright © 2021. This article is distributed under the Creative Commons Attribution License [CC BY].)

phenomenon might relate to an ovarian resistance to the gonadotropin regimen utilized, possibly due to a specific genotype-based mechanism involving polymorphisms of gonadotropins and their receptors. For instance, FSHR c.2039 A>G carriers are less responsive to ovarian stimulation, with fewer oocytes retrieved than asparagine carriers. Moreover, women homozygous for the FSHR-29 G>A (rs1394205) variant genotype have a lower number of oocytes retrieved than those with the GG genotype [28].

In clinical practice, low FOI values should prompt the assessment of possible causative factors for poor/suboptimal response, including ovarian resistance to gonadotropin stimulation related to genetic abnormalities involving gonadotropins and/or their receptors, low gonadotropin starting dose, asynchronous follicular development, and issues associated with triggering of final oocyte maturation and/oocyte pickup (Figure 53.13). Although FOI values <50% likely indicate that the available antral follicles

were not adequately recruited, the percentile/quartile-based definition of FOI normalcy has not been defined yet.

Although we still need more data about the FOI in patients undergoing ovarian stimulation, it has been speculated that strategies to improve the FOI and possibly overcome a suboptimal response to ovarian stimulation, like increasing the FSH starting dose, use of LH supplementation, and dual trigger, could be used in routine practice.

Management strategies and outcomes

The medical management of low-prognosis patients remains incredibly challenging. However, developing and validating a standardized classification system, such as the POSEIDON criteria, underlining fundamental differences related to a poor or suboptimal treatment outcome in terms of oocyte quality and quantity, is

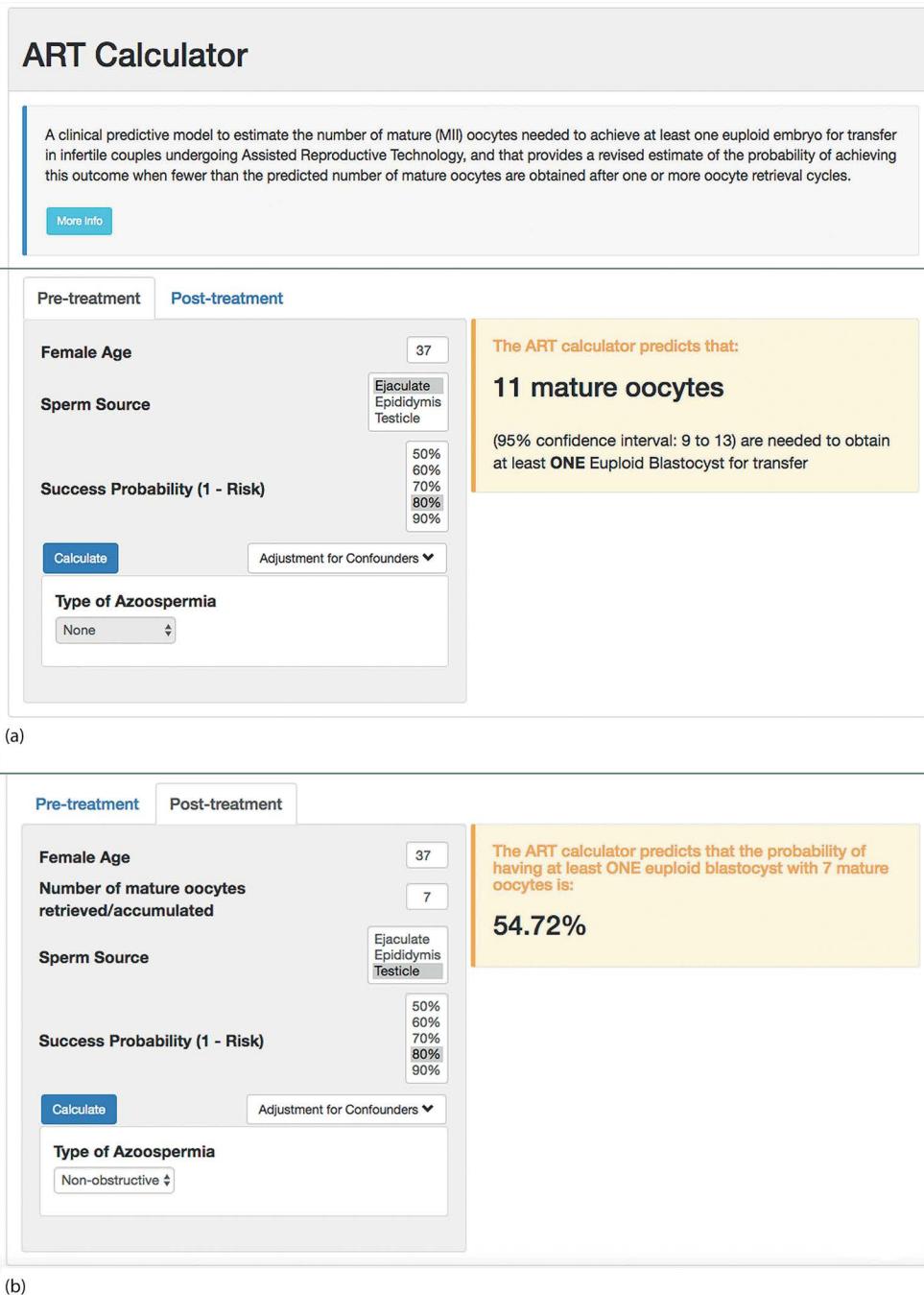


FIGURE 53.11 ART calculator. Online calculator to estimate the minimum number of mature oocytes required to obtain at least one euploid blastocyst for transfer in infertile patients undergoing IVF/ICSI cycles. The figure shows how the calculator can be used in an office-based setting. (a) Pre-treatment, clinicians should input the patient's age and the sperm source for IVF/ICSI. If the option "Testicle" is marked, then the type of azoospermia should also be defined. The user sets the probability of success for the estimation, which indicates the chance of having one or more euploid blastocyst when the predicted number of mature oocytes is achieved. The probability of success complement is the risk, that is, the chance of having no (zero) euploid blastocysts when the predicted number of oocytes is achieved. Once the button "calculate" is pressed, a text box will pop up on the right side of the screen, indicating the predicted minimum number of mature oocytes needed for obtaining at least one euploid blastocyst, with its 95% confidence interval. (b) Post-treatment, i.e. when fewer than the predicted number of mature oocytes are obtained after one or more oocyte retrieval cycles. Clinicians should input the pre-treatment information and the actual number of mature oocytes collected or accumulated. The user sets the probability of success; it reflects the chance of correct estimation according to the exact number of oocytes obtained. Once the button "calculate" is pressed, a text box will pop up on the right side of the screen, indicating the predicted probability of achieving one or more euploid blastocyst with the number of mature oocytes available. The ART calculator can be found online at <http://www.members.groupposeidon.com/Calculator/>. (Reprinted from [24], Copyright © 2021. This article is distributed under the Creative Commons Attribution License [CC BY].)

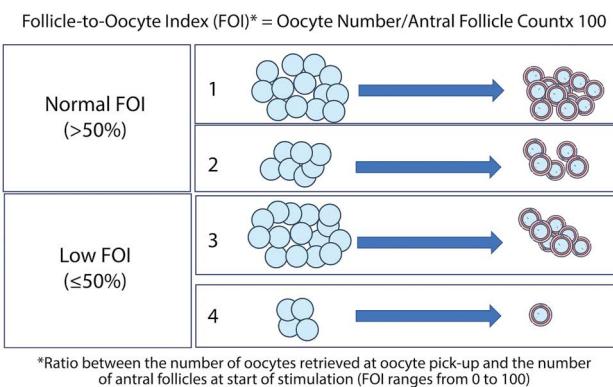


FIGURE 53.12 Follicle-to-oocyte index (FOI). Ovarian sensitivity to stimulation with exogenous gonadotropins as determined using the follicle-to-oocyte index (FOI). Case number 1 depicts a patient with normal FOI, in whom the number of oocytes retrieved was consistent with the AFC at the start of stimulation (FOI ~90%). Case number 2 illustrates a patient who, despite having a suboptimal number of oocytes retrieved (between four and nine), had an FOI of ~70%, thus suggesting that the initial antral follicle cohort was adequately exploited. Case number 3 shows a patient with hypo-response and a suboptimal oocyte number. This patient had only seven oocytes collected despite an AFC of 15 at the beginning of stimulation (FOI ~45%), suggesting that the initial antral follicle cohort was not adequately exploited. Case number 4 depicts a patient with hypo-response and poor response (FOI ~25%), suggesting that the initial follicle cohort was not exploited adequately despite a low ovarian reserve. (Adapted from [13]. Copyright © 2018. This article is distributed under the Creative Commons Attribution License [CC BY].)

a tremendous step forward to create more homogenous groups of patients for clinical management and research. The current evidence concerning treatment strategies for POSEIDON is limited until now, but apparently, these patients could be treated more efficiently, as discussed in the following sections.

Unexpected poor and suboptimal responders (Groups 1 and 2): Treatment rationale, strategies, and outcomes

POSEIDON groups 1 and 2 patients respond poorly (fewer than four oocytes retrieved) or suboptimally (four to nine oocytes retrieved) to standard ovarian stimulation with gonadotropins despite having adequate pre-stimulation ovarian reserve markers [19, 21]. The reasons explaining this phenomenon are not fully understood. Still, it has been suggested that a polygenic trait involving gonadotropins and/or their receptors or the use of sub-optimal ovarian stimulation regimens are involved [13, 27].

Pharmacological interventions have been proposed to improve the FOI and number of oocytes ultimately obtained in patients classified as POSEIDON groups 1 and 2 [27]. Figure 53.14 provides a snapshot of the treatment options available for POSEIDON patients of groups 1 and 2.

While the proposed therapies shown in Figure 53.14 need validation, trials exploring pharmacological interventions for these patients of groups 1 and 2 have emerged recently. The characteristics of existing studies and outcomes are briefly discussed next.

In a 2018 case-control study by Drakopoulos et al., in 160 women with good ovarian reserve markers aged <40 years who responded suboptimally (four to nine oocytes retrieved) after standard ovarian stimulation with FSH, significantly more oocytes were retrieved, and embryos were obtained by increasing the daily FSH dose in a subsequent ovarian stimulation. The mean difference in the number of oocytes between the first and second cycles was 3.2 ($p < 0.05$) [28]. A logistic regression analysis revealed that the FSH dose increment was the only relevant predictor of the number of oocytes retrieved in the subsequent IVF cycle. However, neither the FOI nor the genotyping profile of the studied patients was available for analysis.

In another case-control study of 10 women aged <35 years with AMH values >1.2 ng/mL who had less than four oocytes retrieved after ovarian stimulation with 150 IU daily doses of recombinant FSH, Eftekhar et al. reported significantly more oocytes retrieved (9.2 ± 6.8 vs 1.9 ± 1.1 ; $p = 0.004$) and embryos obtained (4.8 ± 2.8 vs 1.3 ± 0.5 ; $p = 0.013$) by performing an ovarian stimulation in the luteal phase of the same cycle. In their study, the luteal phase stimulation was adjusted by increasing the gonadotropin dose to 300 IU/day and using an hCG trigger (vs GnRH-a trigger used in the follicular phase stimulation) [30].

Two observational studies from China evaluated the effectiveness of progestin-primed ovarian stimulation (vs classic flexible GnRH antagonist protocol) in patients stratified according to POSEIDON groups. Among patients of groups 1 and 2, no significant differences were noted in the number of oocytes retrieved and cumulative live birth rates per aspiration cycle [31, 32]. The authors postulated that progestin-primed stimulation is more patient friendly, as it eliminates GnRH antagonist injections.

In an observational study by Li et al. in Chinese women who had undergone a total of 3342 IVF/ICSI cycles and were stratified under the POSEIDON criteria, significantly more oocytes were obtained in group 1 ($n = 1326$ cycles) and 2 ($n = 767$ cycles) patients after ovarian stimulation using the ultra-long GnRH agonist protocol than with the classic GnRH agonist or GnRH antagonist protocols [33]. In this study, live birth rates per transfer were higher after the ultra-long GnRH agonist protocol in group 1 patients, although no significant differences were noted in group 2.

In another observation study by Cozzolino et al., including 1519 POSEIDON group 2 European women, the authors compared minimal versus conventional ovarian stimulation and reported comparable cumulative live births per started cycle. However, in their study, significantly more oocytes, MII oocytes, and embryos were obtained with the conventional stimulation [34]. Lastly, in a case-control study by Farimani et al., including 96 women stratified according to the POSEIDON criteria and undergoing ovarian stimulation according to the Shanghai protocol, significantly more oocytes were retrieved in the second stimulation after intraovarian platelet-rich plasma injection carried out during the first oocyte pickup in groups 1 and 2 patients ($p < 0.05$) [35].

Although the preceding evidence is preliminary, it seems sound to speculate that adjustments in ovarian stimulation regimens might be of clinical utility to unexpected poor/suboptimal responders under the POSEIDON criteria.

Expected poor responders (groups 3 and 4): Treatment rationale, strategies, and outcomes

Patients of groups 3 and 4 are characterized by having a poor ovarian reserve [1, 2]. Figure 53.15 depicts the proposed treatment strategies for these patients [36].

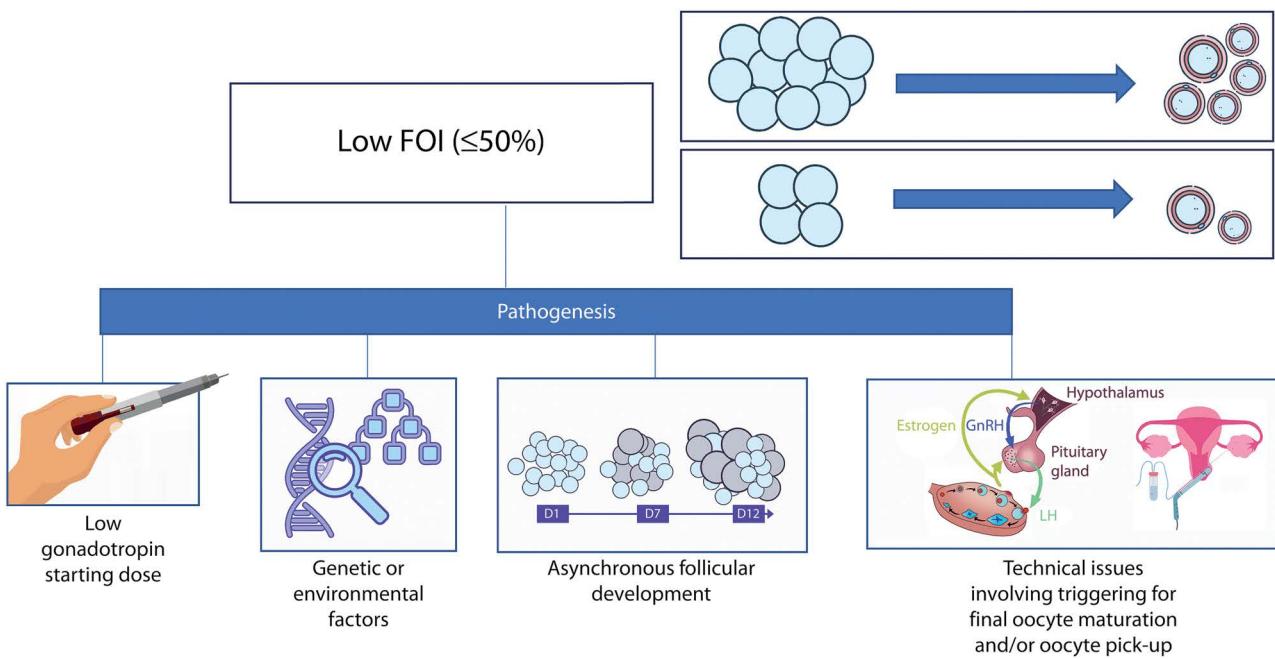


FIGURE 53.13 Possible causative factors for low follicle-to-oocyte indices. The figure depicts the main factors possibly associated with an inadequate FOI value. These factors are not mutually exclusive and include (i) low gonadotropin starting dose, (ii) ovarian hypo-response to gonadotropin stimulation due to genetic variants (e.g. polymorphisms) involving gonadotropins and/or their receptors, (iii) asynchronous follicular development, and (iv) factors related to the trigger of the final oocyte maturation (e.g. medication errors, low dose or inadequate regimen) and/or oocyte pickup (e.g. technical problems related to pump vacuum pressure or needle system, technical difficulties to puncture the follicles, training/expertise). (Adapted from [13]. Copyright © 2018. This article is distributed under the terms of the Creative Commons Attribution License [CC BY].)

Similar to groups 1 and 2, the proposed treatment strategies for groups 3 and 4 need validation, but recent trials have explored the role of interventions for these patients. The characteristics of existing studies and outcomes are briefly discussed here.

In a 2018 randomized controlled trial, Xu et al. investigated the role of coenzyme Q10 used before ovarian stimulation for 60 days in 186 POSEIDON group 3 patients [37]. In their study, the use of 200 mg thrice daily of coenzyme Q10 for 60 days preceding the IVF cycle was associated with an increased number of retrieved oocytes, fertilization rate, and high-quality embryos than non-treated women. In a 2019 observational trial including POSEIDON groups 3 and 4 patients, Cai and co-authors showed that the use of growth hormone from the early follicular phase of the previous menstrual cycle until the oocyte pickup was associated with reduced miscarriage rates and increased live birth rates per transfer in group 4 patients [38].

In a 2020 observational cohort study including POSEIDON group 4 patients, Chen et al. found that individuals pre-treated with dehydroepiandrosterone (DHEA) for 12 weeks achieved higher oocyte yields and embryo numbers than those who were not treated, and these improvements were associated with higher pregnancy rates [39].

Three observational studies compared the GnRH antagonist protocol to the GnRH agonist protocol in POSEIDON groups 3 and 4 patients with conflicting results. In a 2018 study, Huang and colleagues found that among patients of group 3, the GnRH agonist protocol was associated with lower embryo transfer cancellation rates (10.2% vs 22.2%, $p = 0.018$), higher implantation

rates (25.3% vs 10.7%, $p = 0.027$), and higher live birth rates per transfer (27.6% vs 13.0%, $p = 0.024$) than the GnRH antagonist protocol [40]. In their study, no significant differences in the preceding parameters were observed in patients of group 4. Similarly, Li et al., in a study already mentioned in the previous section, showed that the ultra-long agonist protocol and the classical long GnRH agonist protocol (vs GnRH antagonist protocol) were associated with an increased oocyte yield in patients of groups 3 and 4. Additionally, the authors reported higher live birth rates per transfer using GnRH agonist protocols (vs GnRH antagonist protocol) in group 3 POSEIDON patients, albeit no differences were observed in group 4 counterparts [33]. Conversely, Liu and co-authors, in a 2021 study, found that the GnRH agonist protocol was associated with higher cumulative delivery rates in group 4 patients, especially those with AMH values ≥ 0.785 ng/dL [41], whereas no differences were noted in group 3 patients. The discrepancy in the results reported earlier might be related to the fact that live birth per fresh embryo transfer was the primary endpoint in the studies of Huang et al. and Li et al. In contrast, cumulative live birth delivery was the endpoint in the study of Liu et al. Moreover, these studies used different gonadotropin products and doses and allowed dose adjustments during treatment, highlighting the need for further research in this field.

The studies of Du et al. and Zhang et al., mentioned in the previous section, evaluating the effectiveness of progestin-primed ovarian stimulation (vs classic flexible GnRH antagonist protocol) in all POSEIDON groups, compared the preceding protocols in the subsets of groups 3 and 4 patients [31, 32]. Similar to groups

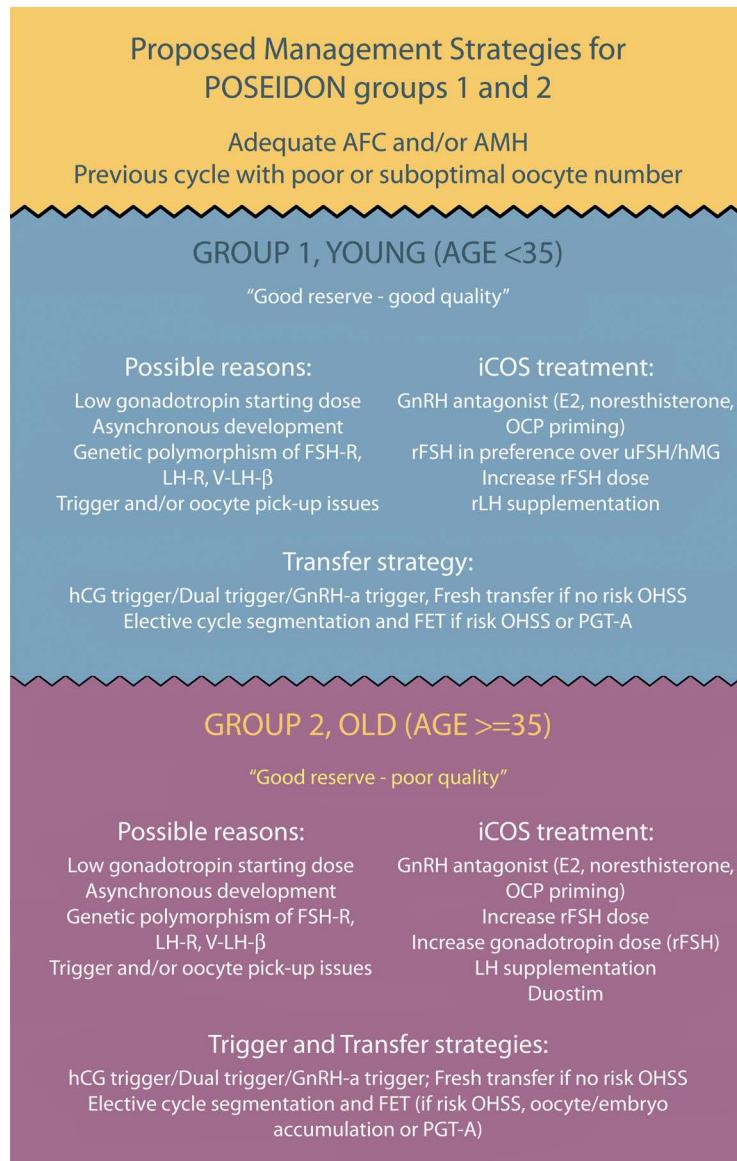


FIGURE 53.14 Proposed management strategies for POSEIDON groups 1 and 2 patients. Patients belonging to groups 1 and 2 share the same common feature of a poor (fewer than four) or suboptimal (four to nine) number of oocytes retrieved after a standard ovarian stimulation with exogenous gonadotropins despite the presence of sufficient ovarian reserve parameters, which independently of age renders them at high risk of a poor reproductive ART outcome. These patients require special attention, especially concerning the optimal use of pharmacological interventions to improve the follicle-to-oocyte index and maximize the number of oocytes retrieved. (a) Pre-treatment: assessing the FOI value of a previous treatment cycle, the minimum number of oocytes required to obtain at least one euploid blastocyst using the ART calculator, and genotyping might help guide the treatment strategy. Pre-treatment with androgens could be considered in patients of subgroups 1a and 1b with good FOI values. In cases of a history of low FOI, pre-treatment, including short-term oestrogen therapy or oral contraceptive pill for synchronization of the follicles before stimulation, may be considered. (b) Ovarian stimulation strategy: in cases of a history of low FOI on a previous stimulation, increased gonadotropin starting doses, adjuvant LH activity during stimulation, and changing trigger strategy to either dual or double trigger should be considered. In general, stimulation should start using GnRH antagonist co-treatment, keeping in mind the possibility of converting to DuoStim to achieve the individualized oocyte number (according to the ART calculator). Otherwise, a long GnRHa protocol should be considered. (c) Ovulation trigger strategy: in the long GnRHa downregulation protocol, hCG is mandatory for the ovulation trigger, whereas GnRHa is mandatory in the follicular phase stimulation of the DuoStim protocol. All trigger agents can be used in the luteal phase stimulation. In non-DuoStim GnRH antagonist cycles, the choice of trigger between GnRHa and hCG should rely on the embryo transfer strategy (fresh or frozen), patient characteristics, and clinical experience. Irrespective of the chosen strategy, it is crucial to remember that the number of oocytes needed to achieve at least one euploid blastocyst for transfer differs between young and older women. (Adapted from [27], Copyright © 2019. This article is distributed under the terms of the Creative Commons Attribution License [CC BY].)

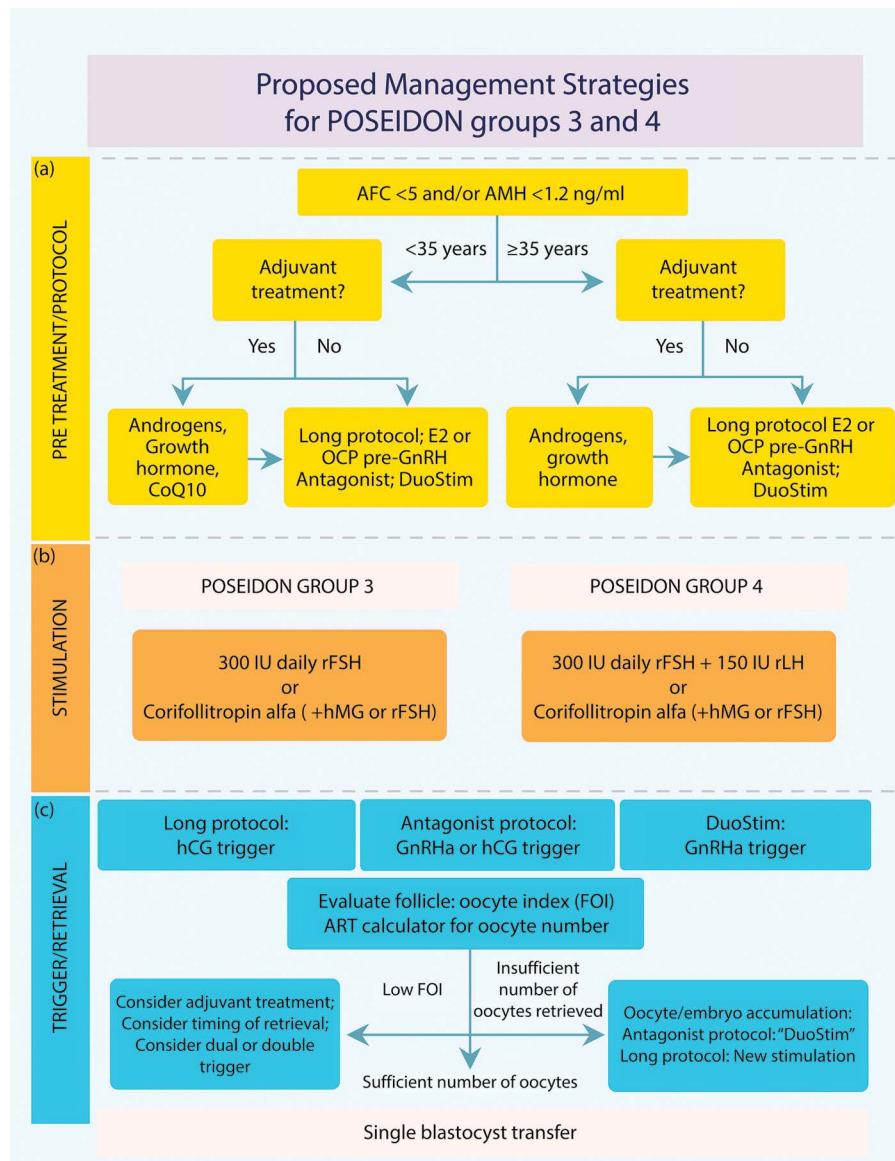


FIGURE 53.15 Proposed management strategies for POSEIDON groups 3 and 4 patients. Patients belonging to groups 3 and 4 share the same common feature of a poor ovarian reserve, which, independently of age, renders them at high risk of a poor reproductive ART outcome. These patients require special attention concerning pre-treatment strategies, ovarian stimulation regimens, adjuvant treatment, and ovulation trigger to optimize the probability of having at least one euploid blastocyst for transfer. (a) Pre-treatment may be considered based on evidence, availability, clinical experience, and patient preference in selected cases. (b) Ovarian stimulation strategy: stimulation should start using GnRH antagonist co-treatment, keeping in mind the possibility of converting to DuoStim to achieve the individualized oocyte number (according to the ART calculator). Otherwise, a long GnRHa protocol should be considered. The first choice in POSEIDON group 3 is the GnRH antagonist cycle with either 300 IU daily of rFSH alone or corifollitropin alfa followed by either rFSH or hMG. In POSEIDON group 4 patients, rLH (75–150 IU daily) could be added from day one of stimulation unless the combination of corifollitropin alfa and hMG was chosen. The GnRH antagonist cycle allows the use of DuoStim, unlike the long-agonist GnRH analogue. (c) Ovulation trigger strategy: in the long GnRHa downregulation protocol, hCG is mandatory as ovulation trigger, whereas GnRHa is mandatory in the follicular phase stimulation of the DuoStim protocol. All trigger agents can be used in the luteal phase stimulation. In non-DuoStim GnRH antagonist cycles, the choice of trigger between GnRHa and hCG should rely on the embryo transfer strategy (fresh or frozen), patient characteristics, and clinical experience. In cases with a history of low FOI, clinicians should consider—for a future treatment cycle—pre-treatment, including short term oestrogen therapy or oral contraceptive pill (OCP) for synchronization of the follicles before stimulation, adjuvant LH activity during stimulation, or changing trigger strategy to either dual or double trigger. If an insufficient number of oocytes is retrieved as determined by the ART calculator, the probability of transferring a euploid embryo should be discussed with the patient to counselling whether an immediate transfer or a new stimulation should be suggested. In all cases, it is crucial to keep in mind that the number of oocytes needed to achieve at least one euploid blastocyst for transfer differs between young and older women. (Adapted from [36], Copyright © 2019. This article is distributed under the terms of the Creative Commons Attribution License [CC BY].)

1 and 2, no significant differences were seen in the number of oocytes retrieved and the cumulative live birth rate per aspiration cycle between treatment regimens. Along these lines, the study of Farimani et al., also mentioned in the previous section, reported an increased number of oocytes retrieved and metaphase II oocytes in the luteal phase stimulation (vs follicular-phase stimulation of the same cycle) in patients of groups 3 and 4 after intraovarian platelet-rich plasma injection—carried out during the first oocyte pickup [35].

Chern et al. reported that the dual trigger (vs hCG trigger) was associated with a higher number of oocytes retrieved and embryos obtained, ultimately resulting in increased live birth rates in POSEIDON group 4 patients [42]. Recently, in 2021, Berker et al. retrospectively evaluated the clinical utility of providing LH-activity supplementation to recombinant FSH stimulation in a cohort of 558 women, consisting mainly of POSEIDON groups 3 and 4 [43]. In their study, the addition of hMG to recombinant FSH from the early follicular phase was associated with higher live birth rates (21.9% vs 11.6%, $p = 0.03$) per initiated cycle than recombinant FSH alone or hMG added from the mid-follicular phase onwards.

In a proof-of-concept study, Orvieto and co-workers used a novel protocol in POSEIDON group 4 patients that combined a modified stop GnRH-agonist protocol with aromatase inhibitor priming to recruit two successive follicular waves while improving follicular sensitivity to FSH [44]. The authors showed that the number of oocytes retrieved was significantly higher (3.8 ± 2.4 vs 2.0 ± 1.2 ; $p = 0.04$) with the novel protocol than the conventional stimulation.

Recently, in 2021, an RCT conducted in China involving group 4 patients showed no benefit of traditional Chinese formula Ding-Kun pill, given twice daily from day five of the previous menstrual cycle until oocyte retrieval, as regards the number of oocytes retrieved and ongoing pregnancy rates [45]. Lastly, Cozzolino et al., in an already mentioned observational study comparing minimally versus conventional ovarian stimulation in POSEIDON group 2 and 4 patients, reported a higher number of oocytes and MII oocytes in group 4 patients treated with the conventional stimulation, albeit no differences were noticed regarding the cumulative live birth rates per started cycle [34]. Notably, in their study, patients received oral contraceptive pills before stimulation.

Collectively, most existing trials are observational and, therefore, not designed to test specific interventions in POSEIDON patients prospectively. The relationship observed between the interventions discussed earlier and improvements in IVF/ICSI outcomes should be seen as associations rather than causal. More research is certainly warranted in this area, particularly in the form of prospective controlled clinical trials.

Case study and practical considerations

Clinicians can take advantage of the POSEIDON classification for individualized treatment planning using a three-step algorithm (Figure 53.16):

1. It should be determined if the patient fits any of the four POSEIDON groups or the fifth group of normal responders (i.e. non-POSEIDON group).
2. The ART calculator can be used to estimate the number of oocytes needed to optimize success.
3. An individualized treatment plan should be designed with the mindset to achieve the estimated oocyte number.

Patient stratification using the POSEIDON criteria	Estimate the minimum number of MII oocytes to have at least one euploid blastocyst for transfer (by ART Calculator)	Patient-oriented treatment strategy aiming to achieve the individualized oocyte number
STEP 1	STEP 2	STEP 3

FIGURE 53.16 Proposed roadmap for managing POSEIDON patients in routine clinical practice. The first step is to determine if the patient fits any of the four POSEIDON groups or the fifth group of normal responders (i.e. non-POSEIDON group). Subsequently, the ART calculator is used to estimate the number of oocytes needed to optimize success. Lastly, an individualized treatment plan is designed to achieve the estimated oocyte number as much as possible.

Of course, the proposed treatment should be discussed with the couple. All aspects are on a shared-decision basis.

The clinical management of POSEIDON group 1 and 2 patients mainly focuses on maximizing the FOI and, consequently, the oocyte and embryo yield, thereby increasing the likelihood of having at least one euploid embryo for transfer [3, 27]. The FOI may be used to determine whether the ovarian reserve was adequately explored during a previous ovarian stimulation [13]. Testing for the presence of common polymorphisms affecting gonadotropins and/or their receptors could also be considered to identify patients at risk of hypo-response [28].

As for POSEIDON group 3 and 4 patients, even though there seem to be limited opportunities for oocyte yield improvement, it is critical to bear in mind that the number of oocytes needed to achieve at least one euploid blastocyst differs between young (group 3) and older (group 4) patients [3, 36]. Therefore, treatment should be tailored accordingly, and an individualized estimation of the minimum number of oocytes needed to obtain at least one euploid embryo may assist counselling and treatment planning [24].

To illustrate how the preceding algorithm works in practice, consider the case of a patient with two previous failed IVF cycles and a poor ovarian reserve treated in the clinic of one of the authors (SE) (Box 53.1).

This patient fits the POSEIDON group 3 based on age, AFC value, and the number of oocytes retrieved in previous cycles. The ART calculator estimated that five MII oocytes would be needed (range four to six) to provide the couple with at least one euploid embryo for transfer [3] (<http://www.members.groupposeidon.com/Calculator/>). The proposed treatment considered her ovarian reserve, which was very low: the DuoStim protocol [46] was used, and six MII oocytes were obtained. Ultimately, three blastocysts were obtained, a singleton live-born at term was delivered after single embryo transfer, and the patient still has two vitrified embryos.

POSORT guidelines

The published literature on the POSEIDON criteria has increased steadily. However, a critical analysis of the existing evidence

BOX 53.1 CASE STUDY

Clinical features

- 33-year-old woman, 35-year-old partner
- Three-year infertility duration; primary infertility
- BMI: 26.1 kg/m² (female); 27.5 kg/m² (male)
- Post-infection tubal obstruction; moderate idiopathic oligo-astheno-teratozoopermia
- Antral follicle count = 4
- No other relevant medical history

History of previous ovarian stimulation and IVF/ICSI outcomes

- Mild stimulation GnRH antagonist protocol, rec-FSH 150 IU/d, hCG trigger: two MII oocytes retrieved; one embryo obtained, day 3 embryo transfer; no pregnancy.
- Ultra-short protocol (hMG 225 IU/d): no oocytes retrieved.

POSEIDON group
Minimum number of MII oocytes estimated by ART calculator
Treatment and outcomes

- Group 3
- Five (95% range: 4–6)
- DuoStim (follicular phase stimulation: rec-FSH + rec-LH 2:1 ratio from stimulation day 1; flexible GnRH antagonist protocol; GnRH-agonist (triptorelin) trigger; luteal phase stimulation: recFSH + rec-LH 2:1 ratio from stimulation day 1; flexible GnRH antagonist protocol; dual trigger (rec-hCG + triptorelin))
- Total of 8 oocytes retrieved (6 MII)
- Three blastocysts developed and vitrified
- Frozen (single) embryo transfer in a hormone replacement cycle without GnRH-a downregulation
- Singleton live-born at term
- Two vitrified blastocysts remaining

Abbreviations: MII, metaphase II; rec-FSH, recombinant follicle-stimulating hormone; rec-LH, recombinant luteinizing hormone; rec-hCG, recombinant human chorionic gonadotropin; hMG, human menopausal gonadotropin; GnRH-a, gonadotropin-releasing hormone agonist; IU, international units; BMI, body mass index.

indicates inconsistent and/or incomplete reporting of essential outcomes in relevant interventional clinical trials. Thus, we developed a guideline based on the best evidence and expert judgment to improve the quality of studies using the POSEIDON criteria [47].

The POSORT (POSEIDON Statement Of Reporting Trials) guideline includes two main parts. In the first part, the critical information to include when reporting trials using the POSEIDON criteria is provided (Table 53.1). The second part offers a list of endpoints—with definitions—relevant to POSEIDON trials (Table 53.2).

Published guidelines like the CONSORT (Consolidated Standards of Reporting Trials), IMPRINT (Improving the Reporting of Clinical Trials of Infertility Treatments), STROBE (Strengthening the Reporting of Observational Studies in Epidemiology), and GRACE (Good Research for Comparative Effectiveness) statements served as guidance to elaborate the list of items shown in Table 53.2.

The goal of the POSORT guideline was to help researchers improve the quality of reporting in studies, apply the POSEIDON criteria, and advance the knowledge concerning the clinical usefulness of the novel classification system to patients, clinicians, and the infertility community. At present, the POSORT guideline is under the stage of dissemination. It is easily and freely accessible online to all who might be interested (<https://www.frontiersin.org/articles/10.3389/fendo.2021.587051/full>).

Conclusions

The POSEIDON criteria underline differences in patient prognosis based on oocyte quantity and quality, creating more homogeneous groups for clinical management and research. Clinical validation using real-world data indicates that (i) POSEIDON patients are typically seen in the fertility clinic; (ii) both AFC and AMH provide acceptable and equivalent accuracy in predicting oocyte yield, further supporting their use and proposed thresholds in daily clinical practice for patient classification, according to the POSEIDON criteria; and (iii) the prognosis of POSEIDON patients undergoing ART, defined by the probability of obtaining a live birth after the transfer of one or more embryos obtained from one aspiration IVF/ICSI cycle, is 50% lower than normal responders and varies across POSEIDON groups, primarily due to age and number of oocytes retrieved.

Furthermore, novel metrics have been introduced to estimate success in ART (i.e. the number of oocytes needed to obtain at least one euploid embryo for transfer) and the dynamic nature of ovarian stimulation with exogenous gonadotropins (FOI). These elements may be used in clinical practice, alongside the POSEIDON classification, to set patient expectations and help guide treatment choices, emphasizing how age, aneuploidy rate, and oocyte number are essential factors for success.

As regards treatment, an individualized treatment plan may be proposed for each POSEIDON group. In groups 1 and 2 patients,

TABLE 53.1 Information to Include when Reporting Studies Using the POSEIDON Criteria*

Title and abstract	Identification as an observational study or randomized trial using the POSEIDON criteria.
Introduction	Explanation of rationale, specific objectives or hypotheses, and how the study may help to advance knowledge concerning the POSEIDON concept.
Methods	
<i>Participants</i>	<ul style="list-style-type: none"> • Inclusion and exclusion criteria must be clearly defined. • Characterize how infertility factors in participants were evaluated, describe the definitions used and the settings where the data were collected. • Define which ovarian marker, AFC or AMH or both, was used to classify the patients as per the POSEIDON criteria, and describe the methods for AFC/AMH measurements. • In POSEIDON groups 1 and 2 studies, previous ovarian stimulation should be characterized. • The preferred unit of analysis is “patient” rather than “cycle.”
<i>Interventions</i>	<ul style="list-style-type: none"> • Characterize the intervention (if applicable) and state the duration of the intervention noting when the treatment started and concluded. State the temporal relation of the intervention to pregnancy.
<i>Outcomes</i>	<ul style="list-style-type: none"> • Clearly define the primary outcome. When more than one embryo transfer cycle occurs, the preferred outcome is cumulative live birth per initiated or aspirated cycle. • Both male and female outcomes, other than cumulative live birth, could be the primary outcome and should be justified. However, when cumulative live birth is not the primary endpoint and embryos are transferred, reproductive outcomes (e.g. live birth rate, ongoing pregnancy rate, miscarriage rate, time to live birth) should be reported. • Efforts should be made to include live birth data, including gestational age, birthweight, and sex of infant. • Clearly define predictors, potential confounders, and effect modifiers. Describe how confounders were adjusted for. • In observational studies, particularly the ones using real-world data, explain features of electronic medical records utilized, including how data quality was verified (e.g. data completeness, availability of data on exposure, outcomes, and covariates). • Describe statistical methods, including those used to control for confounders, sensitivity analyses, and how the sample size was determined.
<i>Data collection and analysis</i>	<ul style="list-style-type: none"> • State the duration of infertility (including whether it is primary or secondary), relevant infertility treatment history, and cause of infertility in women and men. • Report the numbers of couples/patients who were screened and eligible, and describe (in observational studies) the proportion of patients fitting each POSEIDON group and those classified as non-POSEIDON. • Report numbers of individuals completing the follow-up and analysed, and consider the use of a flow diagram. • Provide unadjusted and confounder-adjusted estimates with precision (e.g. 95% confidence interval), and other analyses carried out (e.g. subgroup and sensitivity analyses). • Report harms[†] or unintended effects in each group (men, women, infants) during treatment (including both male and female partners), during pregnancy, and around birth, and in infants after birth.
Results	<ul style="list-style-type: none"> • Discuss generalizability of the study findings and how the results compare to other studies using the POSEIDON concept. • Discuss trial limitations, including but not limited to potential bias and imprecision (factors and interventions affecting endpoints should be discussed as “associations” rather than “causation” in observational studies).
Discussion	

* We recommend application of these guidelines in conjunction with the CONSORT, IMPRINT, STROBE, and GRADE guidelines as appropriate (see <http://www.consort-statement.org/>; <https://stroke-statement.org/>; <https://www.graceprinciples.org/>)

[†] Reportable harms include OHSS, infection, bleeding, multiple pregnancy, and maternal pregnancy complications, and harms or unintended effects on the fetus/new-born, including congenital abnormalities, and major neonatal complications as well as infant developmental delays or medical problems.

Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone.

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who are characterized by having a poor or suboptimal ovarian response despite adequate ovarian markers, it is suggested that treatment strategies focus primarily on pharmacological interventions to optimize ovarian response and FOI, thereby increasing the number of oocytes retrieved. By contrast, in groups 3 and 4 patients, characterized by having a poor ovarian reserve, treatment should be planned to explore ways to increase oocyte number and embryos through accumulation strategies and the possible enhancement of oocyte/embryo quality. In all cases, it is crucial to keep in mind that the number of oocytes needed to achieve at least

one euploid blastocyst for transfer differs between young and older women. Further randomized and pragmatic clinical trials and large real-world data observational studies are warranted to explore the clinical utility of various interventions to improve the reproductive outcomes of the POSEIDON patient. In this regard, the POSORT guideline may help researchers improve the quality of reporting.

The ultimate goal of the POSEIDON group is to advance the knowledge concerning the management of infertility patients undergoing ART, with tangible benefits to patients, clinicians, and the infertility community as a whole.

TABLE 53.2 Endpoints in Clinical Trials Using the POSEIDON Criteria

Endpoint	Definition	Priority
Cumulative live birth delivery rate (CDR) ^a	Number of deliveries with at least one live birth resulting from one initiated, aspirated, or embryo transfer ART cycle, including all cycles in which fresh and/or frozen embryos are transferred, until one delivery with a live birth occurs or until all embryos are used, whichever occurs first, expressed per 100 cycles (denominator must be specified, i.e. initiated or aspirated cycles)	Highly recommended
Time to pregnancy/time to live birth (TTP/TTLB)	The time taken to establish a clinical pregnancy or live birth, measured in days or in number of treatment cycles (e.g. start time point from oocyte retrieval and end time point the day of delivery)	Optional
Follicle-to-oocyte index (FOI)	Ratio between the number of oocytes retrieved at oocyte pickup and the number of antral follicles (AFC) at the start of stimulation	Recommended
Number of oocytes retrieved	Total number of oocytes retrieved after oocyte pickup	Highly recommended
Number of metaphase II oocytes	Total number of metaphase II oocytes retrieved after oocyte pickup	Highly recommended
Number of embryos generated	Total number of viable embryos ^b generated after an IVF or ICSI cycle	Highly recommended
Percentage of patients who achieved the minimum number of metaphase II oocytes estimated by the ART calculator	The ART calculator is a clinical predictive model that estimates, prior to treatment, the minimum number of metaphase II oocytes (MIImin) (and the 95% confidence interval of that number) needed to obtain at least one euploid blastocyst ^c	Optional
Prevalence of low prognosis (POSEIDON) and non-low prognosis (non-POSEIDON)	Frequency (%) of POSEIDON patients (by subgroup) and non-POSEIDON patients in the cohort ^d	Highly recommended
Live birth delivery rate (LBR) ^a	Number of deliveries that resulted in at least one live birth, expressed per 100 cycle attempts (initiated, aspirated, transfer cycles)	Recommended
Ongoing Pregnancy rate (OPR)	Number of viable intrauterine pregnancies of at least 12 weeks duration confirmed on ultrasound scan per 100 clinical pregnancies	Optional
Multiple birth rate	Number of multiple births, defined by the complete expulsion or extraction of ≥1 fetus, after ≥ 22 weeks gestational age (e.g. twin delivery = two births) per 100 deliveries	Optional
Miscarriage rates	Number of spontaneous losses of clinical pregnancies before 22 completed weeks of gestational age per 100 clinical pregnancies	Recommended

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Notes: ^aLive birth: any delivery of a live infant ≥22 weeks' gestation (fetus exiting the body with signs of life: movement, breathing, heartbeat).

^b The embryo stage must be specified (cleavage, blastocyst).

^c The probability of success (e.g., 70%, 80%, and 90%) used for the estimation should be specified.

^d Observational studies, including real-world data analysis.

Abbreviations: AFC, antral follicle count; ART, assisted reproductive technology; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

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References

- Alviggi C, Andersen CY, Buehler K, Conforti A, De Placido G, Esteves SC, Fischer R, Galliano D, Polyzos NP, Sunkara SK, Ubaldi FM, Humaidan P, POSEIDON Group (Patient-Oriented Strategies Encompassing Individualized Oocyte Number). A new more detailed stratification of low responders to ovarian stimulation: From a poor ovarian response to a low prognosis concept. Fertil Steril. 2016;105(6):1452–3. doi: [10.1016/j.fertnstert.2016.02.005](https://doi.org/10.1016/j.fertnstert.2016.02.005).
- Humaidan P, Alviggi C, Fischer R, Esteves SC. The novel POSEIDON stratification of 'Low prognosis patients in assisted reproductive Technology' and its proposed marker of successful outcome. F1000Res. 2016;5:2911. doi: [10.12688/f1000research.10382.1](https://doi.org/10.12688/f1000research.10382.1).
- Esteves SC, Alviggi C, Humaidan P, Fischer R, Andersen CY, Conforti A, Bühler K, Sunkara SK, Polyzos NP, Galliano D, Grynberg M, Yarali H, Özbeş IY, Roque M, Vuong LN, Banker M, Rienzi L, Vaiarelli A, Cimadomo D, Ubaldi FM. The POSEIDON criteria and its measure of success through the eyes of clinicians and embryologists. Front Endocrinol (Lausanne). 2019 Nov 20;10:814. doi: [10.3389/fendo.2019.00814](https://doi.org/10.3389/fendo.2019.00814).
- Drakopoulos P, Bardhi E, Boudry L, Vaiarelli A, Makrigiannakis A, Esteves SC, Tournaye H, Blockeel C. Update on The management of poor ovarian response in IVF: The shift from Bologna criteria to The POSEIDON concept. Ther Adv Reprod Health. 2020;14:2633494120941480. doi: [10.1177/2633494120941480](https://doi.org/10.1177/2633494120941480).
- Esteves SC, Carvalho JE, Martinhago CD, Melo AA, Bento FC, Humaidan P, Alviggi C; POSEIDON (Patient-Oriented Strategies Encompassing Individualized Oocyte Number) Group. Estimation of age-dependent decrease in blastocyst euploidy by next-generation sequencing: Development of a novel prediction model. Panminerva Med. 2019;61(1):3–10. doi: [10.23736/S0031-0808.18.03507-3](https://doi.org/10.23736/S0031-0808.18.03507-3).
- Tal R, Seifer DB. Ovarian reserve testing: A user's guide. Am J Obstet Gynecol. 2017;217(2):129–40. doi: [10.1016/j.ajog.2017.02.027](https://doi.org/10.1016/j.ajog.2017.02.027).
- Broekmans FJ, Kwee J, Hendriks DJ, Mol BW, Lambalk CB. A systematic review of tests predicting ovarian reserve and IVF

- outcome. *Hum Reprod Update*. 2006;12(6):685–718. doi: [10.1093/humupd/dml034](https://doi.org/10.1093/humupd/dml034).
- 8. Broer SL, Mol BW, Hendriks D, Broekmans FJ. The role of antimüllerian hormone in prediction of outcome after IVF: Comparison with the antral follicle count. *Fertil Steril*. 2009;91(3):705–14. doi: [10.1016/j.fertnstert.2007.12.013](https://doi.org/10.1016/j.fertnstert.2007.12.013).
 - 9. Grisendi V, Mastellari E, La Marca A. Ovarian reserve markers to identify poor responders in the context of POSEIDON classification. *Front Endocrinol (Lausanne)*. 2019;10:281.
 - 10. La Marca A, Sunkara SK. Individualization of controlled ovarian stimulation in IVF using ovarian reserve markers: From theory to practice. *Hum Reprod Update*. 2014;20:124–40.
 - 11. Nelson SM. Biomarkers of ovarian response: Current and future applications. *Fertil Steril*. 2013;99:963–9.
 - 12. Drakopoulos P, Blockeel C, Stoop D, Camus M, de Vos M, Tournaye H, Polyzos NP. Conventional ovarian stimulation and single embryo transfer for IVF/ICSI. How many oocytes do we need to maximize cumulative live birth rates after utilization of all fresh and frozen embryos? *Hum Reprod*. 2016;31(2):370–6. doi: [10.1093/humrep/dev316](https://doi.org/10.1093/humrep/dev316).
 - 13. Alviggi C, Conforti A, Esteves SC, Vallone R, Venturella R, Staiano S, Castaldo E, Andersen CY, De Placido G. Understanding ovarian hypo-response to exogenous gonadotropin in ovarian stimulation and its new proposed marker—the follicle-to-oocyte (FOI) index. *Front Endocrinol*. 2018;9:589. doi: [10.3389/fendo.2018.00589](https://doi.org/10.3389/fendo.2018.00589).
 - 14. Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, et al. The international glossary on infertility and fertility care, 2017. *Fertil Steril*. 2017;108(3):393–406. doi: [10.1016/j.fertnstert.2017.06.005](https://doi.org/10.1016/j.fertnstert.2017.06.005).
 - 15. Moragianni VA, Penzias AS. Cumulative live-birth rates after assisted reproductive technology. *Curr Opin Obstet Gynecol*. 2010;22(3):189–92. doi: [10.1097/GCO.0b013e328338493f](https://doi.org/10.1097/GCO.0b013e328338493f).
 - 16. Maheshwari A, McLernon D, Bhattacharya S. Cumulative live birth rate: Time for a consensus? *Hum Reprod*. 2015;30(12):2703–7. doi: [10.1093/humrep/dev263](https://doi.org/10.1093/humrep/dev263).
 - 17. Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, Kolibianakis E, Kunicki M, La MA, Lainas G, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI. *Hum Reprod Open*. 2020;2020:hoaa009. Erratum in: *Hum Reprod Open* 2020; 2020:hoaa067.
 - 18. Malizia BA, Hacker MR, Penzias AS. Cumulative live-birth rates after in vitro fertilization. *N Engl J Med*. 2009;360(3):236–43. doi: [10.1056/NEJMoa0803072](https://doi.org/10.1056/NEJMoa0803072).
 - 19. Esteves SC, Yarali H, Vuong LN, Carvalho JF, Özbeş İY, Polat M, Le HL, Pham TD, Ho TM. Low prognosis by the POSEIDON criteria in women undergoing assisted reproductive technology: A multicenter and multinational prevalence study of over 13,000 patients. *Front Endocrinol (Lausanne)*. 2021;12:630550. doi: [10.3389/fendo.2021.630550](https://doi.org/10.3389/fendo.2021.630550).
 - 20. Esteves SC, Yarali H, Vuong LN, Carvalho JF, Özbeş İY, Polat M, Le HL, Pham TD, Ho TM. Antral follicle count and anti-Müllerian hormone to classify low-prognosis women under the POSEIDON criteria: A classification agreement study of over 9000 patients. *Hum Reprod*. 2021;36(6):1530–41. doi: [10.1093/humrep/deab056](https://doi.org/10.1093/humrep/deab056).
 - 21. Esteves SC, Yarali H, Vuong LN, Carvalho JF, Özbeş İY, Polat M, Le HL, Pham TD, Ho TM, Humaidan P, Alviggi C. Cumulative delivery rate per aspiration IVF/ICSI cycle in POSEIDON patients: A real-world evidence study of 9073 patients. *Hum Reprod*. 2021;36(8):2157–69. doi: [10.1093/humrep/deab152](https://doi.org/10.1093/humrep/deab152).
 - 22. Sciorio R, Esteves SC. Clinical utility of freeze-all approach in ART treatment: A mini-review. *Cryobiology*. 2020;92:9–14. doi: [10.1016/j.cryobiol.2019.11.041](https://doi.org/10.1016/j.cryobiol.2019.11.041).
 - 23. Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B, Treff NR, Scott RT Jr. In vitro fertilization with single euploid blastocyst transfer: A randomized controlled trial. *Fertil Steril*. 2013;100(1):100–7.e1. doi: [10.1016/j.fertnstert.2013.02.056](https://doi.org/10.1016/j.fertnstert.2013.02.056).
 - 24. Esteves SC, Carvalho JC, Bento FC, Santos J. A novel predictive model to estimate the number of mature oocytes required for obtaining at least one euploid blastocyst for transfer in couples undergoing in vitro fertilization/intracytoplasmic sperm injection: The ART calculator. *Front Endocrinol*. 2019;10:99. doi: [10.3389/fendo.2019.00099](https://doi.org/10.3389/fendo.2019.00099).
 - 25. Esteves SC, Yarali H, Ubaldi FM, Carvalho JF, Bento FC, Vaiarelli A, Cimadomo D, Özbeş İY, Polat M, Bozdag G, Rienzi L, Alviggi C. Validation of ART calculator for predicting the number of metaphase II oocytes required for obtaining at least one euploid blastocyst for transfer in couples undergoing in vitro fertilization/intracytoplasmic sperm injection. *Front Endocrinol (Lausanne)*. 2020;10:917. doi: [10.3389/fendo.2019.00917](https://doi.org/10.3389/fendo.2019.00917).
 - 26. Chen L, Wang H, Zhou H, Bai H, Wang T, Shi W, Shi J. Follicular output rate and follicle-to-oocyte index of low prognosis patients according to POSEIDON criteria: A retrospective cohort study of 32,128 treatment cycles. *Front Endocrinol (Lausanne)*. 2020;11:181. doi: [10.3389/fendo.2020.00181](https://doi.org/10.3389/fendo.2020.00181).
 - 27. Conforti A, Esteves SC, Cimadomo D, Vaiarelli A, Rella D, Ubaldi F, Zullo FM, Placido D, Alviggi G C. Management of women with an unexpected low ovarian response to gonadotropin. *Front Endocrinol (Lausanne)*. 2019;10:387. doi: [10.3389/fendo.2019.00387](https://doi.org/10.3389/fendo.2019.00387).
 - 28. Alviggi C, Conforti A, Santi D, Esteves SC, Andersen CY, Humaidan P, Chiodini P, De Placido G, Simoni M. Clinical relevance of genetic variants of gonadotrophins and their receptors in controlled ovarian stimulation: A systematic review and meta-analysis. *Hum Reprod*. 2018;24(5):599–614. doi: <https://doi.org/10.1093/humupd/dmy019>.
 - 29. Drakopoulos P, Santos-Ribeiro S, Bosch E, Garcia-Velasco J, Blockeel C, Romito A, Tournaye H, Polyzos NP. The effect of dose adjustments in a subsequent cycle of women with suboptimal response following conventional ovarian stimulation. *Front Endocrinol (Lausanne)*. 2018;9:361. doi: [10.3389/fendo.2018.00361](https://doi.org/10.3389/fendo.2018.00361).
 - 30. Eftekhar M, Mohammadi B, Khani P, Lahijani MM. Dual stimulation in unexpected poor responder POSEIDON classification group 1, sub-group 2a: A cross-sectional study. *Int J Reprod Biomed*. 2020;18(6):465–70. doi: [10.18502/ijrm.v13i6.7287](https://doi.org/10.18502/ijrm.v13i6.7287).
 - 31. Du M, Zhang J, Li Z, Liu X, Li J, Liu W, Guan Y. Comparison of the cumulative live birth rates of progestin-primed ovarian stimulation and flexible GnRH antagonist protocols in patients with low prognosis. *Front Endocrinol (Lausanne)*. 2021;12:705264. doi: [10.3389/fendo.2021.705264](https://doi.org/10.3389/fendo.2021.705264).
 - 32. Zhang S, Yin Y, Li Q, Zhang C. Comparison of cumulative live birth rates between GnRH-a and PPOS in low-prognosis patients according to POSEIDON criteria: a cohort study. *Front Endocrinol (Lausanne)*. 2021;12:644456. doi: [10.3389/fendo.2021.644456](https://doi.org/10.3389/fendo.2021.644456).
 - 33. Li F, Ye T, Kong H, Li J, Hu L, Jin H, Su Y, Li G. Efficacies of different ovarian hyperstimulation protocols in poor ovarian responders classified by the POSEIDON criteria. *Aging*. 2020;12(10):9354–64. doi: <https://doi.org/10.18632/aging.103210>.
 - 34. Cozzolino M, Cecchino GN, Bosch E, Garcia-Velasco JA, Garrido N. Minimal ovarian stimulation is an alternative to conventional protocols for older women according to POSEIDON’s stratification: A retrospective multicenter cohort study. *J Assist Reprod Genet*. 2021;38(7):1799–807. doi: [10.1007/s10815-021-02185-2](https://doi.org/10.1007/s10815-021-02185-2).
 - 35. Farimani M, Nazari A, Mohammadi S, Anvari Aliabad R. Evaluation of intra-ovarian platelet-rich plasma administration on oocytes-dependent variables in patients with poor ovarian response: A retrospective study according to the POSEIDON criteria. *Reprod Biol Endocrinol*. 2021;19(1):137. doi: [10.1186/s12958-021-00826-w](https://doi.org/10.1186/s12958-021-00826-w).
 - 36. Haahr T, Dosouto C, Alviggi C, Esteves SC, Humaidan P. Management strategies for POSEIDON groups 3 and 4. *Front Endocrinol*. 2019;10:614. doi: [10.3389/fendo.2019.00614](https://doi.org/10.3389/fendo.2019.00614).
 - 37. Xu Y, Nisenblat V, Lu C, Li R, Qiao J, Zhen X, Wang S. Pretreatment with coenzyme Q10 improves ovarian response and embryo quality in low-prognosis young women with decreased ovarian reserve: A randomized controlled trial. *Reprod Biol Endocrinol*. 2018;16(1):29. doi: [10.1186/s12958-018-0343-0](https://doi.org/10.1186/s12958-018-0343-0).

38. Cai MH, Gao LZ, Liang XY, Fang C, Wu YQ, Yang X. The effect of growth hormone on the clinical outcomes of poor ovarian reserve patients undergoing in vitro fertilization/intracytoplasmic sperm injection treatment: A retrospective study based on POSEIDON criteria. *Front Endocrinol (Lausanne)*. 2019;10:775. doi: [10.3389/fendo.2019.00775](https://doi.org/10.3389/fendo.2019.00775).
39. Chen SN, Tsui KH, Wang PH, Chern CU, Wen ZH, Lin LT. Dehydroepiandrosterone supplementation improves the outcomes of in vitro fertilization cycles in older patients with diminished ovarian reserve. *Front Endocrinol (Lausanne)* 2019;10:800. doi: [10.3389/fendo.2019.00800](https://doi.org/10.3389/fendo.2019.00800).
40. Huang MC, Tzeng SL, Lee CI, Chen HH, Huang CC, Lee TH, Lee MS. GnRH agonist long protocol versus GnRH antagonist protocol for various aged patients with diminished ovarian reserve: A retrospective study. *PloS One*. 2018;13(11):e0207081. doi: <https://doi.org/10.1371/journal.pone.0207081>.
41. Liu L, Xu Y, Huang J, Zhou C. Patients with higher anti-Müllerian hormone levels from POSEIDON group 4 benefit from GnRH-agonist long protocol: A retrospective study. *Eur J Obstet Gynecol Reprod Biol*. 2021;257:88–94. doi: [10.1016/j.ejogrb.2020.12.024](https://doi.org/10.1016/j.ejogrb.2020.12.024).
42. Chern CU, Li JY, Tsui KH, Wang PH, Wen ZH, Lin LT. Dual-trigger improves the outcomes of in vitro fertilization cycles in older patients with diminished ovarian reserve: A retrospective cohort study. *PloS One*. 2020;15(7):e0235707. doi: <https://doi.org/10.1371/journal.pone.0235707>.
43. Berker B, Şükür YE, Özdemir E, Özmen B, Sönmezler M, Atabekoğlu CS, Aytaç R. Human menopausal gonadotropin commenced on early follicular period increases live birth rates in POSEIDON group 3 and 4 poor responders. *Reprod Sci*. 2021;28(2):488–94. doi: [10.1007/s43032-020-00300-9](https://doi.org/10.1007/s43032-020-00300-9).
44. Orvieto R, Nahum R, Aizer A, Haas J, Kirshenbaum M. A novel stimulation protocol for poor-responder patients: Combining the stop GnRH-ag protocol with letrozole priming and multiple-dose GnRH-ant: A proof of concept. *Gynecol Obstet Invest*. 2021;86(1-2):149–54. doi: [10.1159/000513669](https://doi.org/10.1159/000513669).
45. Song JY, Gao DD, Cao XL, Xiang S, Chen YH, Teng YL, Li XF, Liu HP, Wang FX, Zhang B, Xu LH, Zhou L, Huang XH, Sun ZG. The role of traditional Chinese formula ding-kun pill (DKP) in expected poor ovarian response women (POSEIDON group 4) undergoing in vitro fertilization-embryo transfer: A multi-center, randomized, double-blind, placebo-controlled trial. *Front Endocrinol (Lausanne)*. 2021;12:675997. doi: [10.3389/fendo.2021.675997](https://doi.org/10.3389/fendo.2021.675997).
46. Vaiarelli A, Cimadomo D, Trabucco E, Vallefuoco R, Buffo L, Dusi L, Fiorini F, Barnocchi N, Bulletti FM, Rienzi L, Ubaldi FM. Double stimulation in the same ovarian cycle (DuoStim) to maximize the number of oocytes retrieved from poor prognosis patients: A multicenter experience and SWOT analysis. *Front Endocrinol (Lausanne)*. 2018;9:317. doi: [10.3389/fendo.2018.00317](https://doi.org/10.3389/fendo.2018.00317).
47. Esteves SC, Conforti A, Sunkara SK, Carbone L, Picarelli S, Vaiarelli A, Cimadomo D, Rienzi L, Ubaldi FM, Zullo F, Andersen CY, Orvieto R, Humaidan P, Alviggi C. Improving reporting of clinical studies using the POSEIDON criteria: POSORT guidelines. *Front Endocrinol (Lausanne)*. 2021;12:587051. doi: [10.3389/fendo.2021.587051](https://doi.org/10.3389/fendo.2021.587051).

CONTROLLED OVARIAN STIMULATION FOR LOW-RESPONDER PATIENTS

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Introduction

The main goal of Assisted Reproduction Technology (ART) is the birth of at least one healthy baby. Yet, whilst from a clinical perspective cumulative live birth rate per intention to treat (CLBR per ITT) has to be considered as the main measure of success, infertile couples also value their investment in terms of time, costs, and energies to realize their parenting plan (which may entail also more than a single child) [1, 2]. In this regard, safety and efficiency are mandatory in modern ART.

For a safe treatment, the following features are critical:

- Personalize the starting dose, the type of gonadotropins, and the controlled ovarian stimulation (COS) protocol.
- Reduce the risk of ovarian hyperstimulation syndrome (OHSS) through GnRH antagonists for COS, GnRH agonist as ovulation trigger and cycle segmentation (i.e. postponement of embryo transfer on a non-stimulated endometrium).
- Reduce the risk of multiple pregnancy by transferring a single embryo, preferably a blastocyst with the highest chance of implantation.

For an efficient treatment, instead, we shall:

- Maximize ovarian response to COS.
- Focus on CLBR.
- Adopt validated lab technologies and clinical strategies for vitrification, blastocyst culture, and embryo selection. Especially, pre-implantation genetic testing for aneuploidies (PGT-A) is critical in advanced maternal age (AMA) women to increase IVF efficiency [3].

Hereafter we cover the main aspects to keep under control to fulfil all these precepts.

The ovarian reserve

To assess the ovarian reserve is the key to predict COS response. This assessment is based on biochemical (FSH and AMH) and morphological parameters (antral follicle count [AFC]). FSH has been widely used in the past but did not show any association with the AFC, that is measured by transvaginal ultrasonography in the early follicular phase and consists of counting the number of antral follicles (<10 mm of average diameter) in the ovaries [4]. ESHRE Guidelines 2019 concluded that AFC and AMH should be considered the most sensitive indicators of the ovarian reserve to be assessed aiming at COS personalization. Nevertheless, both these parameters suffer from important limitations: AFC is subject to high inter-operator variability, whereas AMH levels are highly variable across kits and laboratories [5].

The importance of the number of oocytes retrieved and age

Sunkara analysed 400,135 IVF cycles and claimed that 15 is the ideal number of oocytes to retrieve after COS to maximize the chance of pregnancy, whereas beyond this number the chance of pregnancy decreases, perhaps due to the high levels of oestrogens and their impact on endometrial receptivity [6]. Similar results have been reported more recently by Polyzos et al. [7]. This information is valuable if entailing only the first fresh transfer without a cumulative perspective that encompasses also all vitrified-warmed ones. From the latter perspective, the higher the number of oocytes retrieved, the higher the CLBR, even beyond 15 [7, 8].

Although the quantity of oocytes retrieved is an important variable, their competence is the most important effector to achieve a healthy live birth. This indicator, though, decreases over time as woman age, increases mainly because of lower blastulation rates after the age of 40 [9] and higher aneuploidy rates after the age of 35, largely imputable to meiotic mis-segregations during the last stages of oogenesis [10–12]. At present, the only strategy to counteract these rates is to collect a larger number of oocytes per ovarian cycle through a personalized COS approach [1, 10].

From ovarian physiology to multiple follicle growth

In the natural cycle, the sudden drop in oestrogen levels, inhibin A, and progesterone during the late luteal phase, which are secondary to the regression of the corpus luteum, results in an increased frequency of pulsatile GnRH secretion, which induces an increase in serum FSH concentrations at the end of the luteal phase. When these concentrations reach critical thresholds, antral follicles between 2 and 4 mm in diameter are recruited and begin their typical growth trajectory [13]. Their number depends on each woman's follicular wealth. In the initial follicular phase, the increase in oestrogen production is responsible for the fall of serum FSH levels below the threshold level. This event reduces the stimulation time of the FSH on the growing follicle pool, resulting in the exclusive growth of the dominant follicle and the atresia of the others [14]. The growth of the dominant follicle, apart from its increased sensitivity to FSH, may also be due to the central role played by LH in follicular selection and dominance. In fact, the granulosa cells of the larger follicles become sensitive to LH because of the expression of LH receptors after the increase in oestrogen and FSH concentration. The growth of these latter follicles is therefore less dependent on FSH and more on LH [15]. Based on these processes, we can conclude that continuously administering exogenous gonadotropins prevents both follicular selection and dominance, thereby allowing the synchronous

development and maturation of all recruited follicles that otherwise would physiologically undergo atresia.

New theories of folliculogenesis

The ovary is an extremely dynamic organ. The traditional theory of human folliculogenesis, which was developed more than 50 years ago, has been in fact challenged. In cattle and other large mammals, the presence of multiple follicle waves within the same menstrual cycle was demonstrated more than 30 years ago. A phenomenon then well documented also in women histologically, ultrasonographically, and endocrinologically [16]. The current theories of folliculogenesis are three [17]:

- *The classical theory*: a single cohort of follicles is recruited solely in the luteal phase of the previous cycle.
- *Continuous recruitment*: waves of follicle development continuously arise and regress in a single menstrual cycle.
- *Multiple waves*: two to three follicular waves arise between one ovulation and another, named “minor” if anovulatory and “major” if ovulatory.

Unfortunately, the mechanisms regulating the “follicle waves” are unclear. From a clinical perspective, this dynamism led to the implementation of three unconventional COS protocols for time-sensitive patients (poor responders, advanced maternal age, and oncologic women):

- *Random start*: COS is started independently of the menstrual cycle at any stage. This regimen is used in fertility preservation protocols to reduce the time to retrieval.
- *Stimulation in the luteal phase*: the gonadotropins are administrated starting between the 17th and 21st day of the cycle. This strategy has been proposed in patients with reduced ovarian reserve.
- *Double stimulation in a single ovarian cycle (DuoStim)*: two stimulations are conducted back-to-back in the same ovarian cycle. This strategy has been suggested to poor-prognosis patients to optimize the chances of live birth in a short timeframe while minimizing the risk of treatment discontinuation.

Controlled ovarian stimulation

Over the past 30 years, several COS protocols have been suggested to maximize ovarian response in poor-prognosis patients. The ideal characteristics of a successful COS protocol are low cancellation rate; reduced costs, risks, and side effects; and limited endocrine and ultrasound monitoring. The main patient characteristics to choose COS are age, ovarian reserve markers, response after previous COS, and body mass index (BMI). Two main regimens exist entailing either GnRH agonists or GnRH antagonists to block the release of pituitary gonadotropins, virtually eliminating the risk of premature LH peaks and consequent premature luteinization [18, 19]. They both led to significant clinical improvements and the possibility of better managing the cycle.

GnRH antagonists prevent premature peaks of LH by binding to pituitary GnRH receptors in a competitive and dose-dependent manner without inducing their activation. Unlike GnRH agonists, this does not cause the release of endogenous gonadotropins before reaching pituitary desensitization, but the immediate

and reversible suppression of gonadotropin secretion. Due to their mechanism of action, GnRH antagonists can only be used in the mid-to-late follicular phase, a period at risk for premature LH peaks, allowing stimulation with gonadotropins to begin in the early follicular phase (second or third day of the cycle), thus acting on the physiological follicular recruitment. This approach allows the action of endogenous FSH to be exploited rather than inhibited, with a consequent reduction in the duration of administration and consumption of exogenous gonadotropins. Although randomized studies have not observed significantly better clinical outcomes when the antagonist is compared with the agonist protocol [20], its non-inferiority and greater simplicity made it a valid therapeutic option in poor responders.

Its advantages [5] are:

- The duration of COS is considerably shorter with respect to the agonist, involving also fewer endocrine controls, a higher patient compliance, and a lower treatment discontinuation.
- The prevalence of ovarian hyperstimulation syndrome (OHSS) is significantly reduced due to lower oestrogen levels and the possibility to postpone embryo transfer (ET).

Its disadvantages [5] are:

- The antagonist suppresses the secretion of endogenous gonadotropins more profoundly than the agonist. Although follicular growth does not seem affected by antagonist administration, some authors consider it appropriate to increase FSH doses and/or administer gonadotropin preparations containing recombinant LH or LH activity. However, the data in the literature are conflicting.
- Protocols with GnRH antagonists offer less flexibility in work organization than long protocols, although some programming is possible through pre-treatment with oral contraceptives or progestogens.

Customization of hormonal stimulation

IVF patients may be classified into four categories based on their predicted response to conventional COS:

1. Patients with a high expected response (more than 15 oocytes retrieved)
2. Patients with normal expected response (from 10 to 15 oocytes retrieved)
3. Patients with suboptimal expected response (from 4 to 9 oocytes retrieved)
4. Patients with expected poor response (fewer than 4 oocytes)

Based on this classification:

- When ovarian response may be excessive, our goal is to minimize OHSS through GnRH antagonist protocols, minimal doses of gonadotropins, induction of oocyte maturation with GnRH analogue, and segmentation of the cycle.
- When normal response is expected, our aim is to maximize success rates by using COS protocols with GnRH agonist or antagonist and choosing a correct dose of gonadotropins to better exploit the ovarian reserve.

- In poor or suboptimal responders, our objective is to use maximum gonadotropins doses (not exceeding 300 IU/day) in an antagonist protocol and/or using strategies to collect the highest possible number of oocytes in a short timeframe.

In the latter category, “double stimulation in a single ovarian cycle” (DuoStim) is a promising approach. Natural cycle or egg donations are also options in case of exhausted ovarian reserve.

In general, the starting dose of exogenous gonadotropins should be between 100 IU and 300–375 IU per day for the first four or five days with recombinant or urinary FSH or purified urinary menotropins according to the patient’s age, ovarian reserve, and the results of previous ovarian stimulations. These starting doses can then be adjusted in due course according to ovarian response. However, ovarian stimulation has two fundamental characteristics: exogenous gonadotropins used during COS only allow the maturation of those follicles selected by the ovary, but they cannot create follicles ex novo. In other terms, increasing the daily dose of gonadotropins is worthless in poor responders [21].

The management of poor prognosis patients

The management of poor responders undergoing COS is very controversial. Inadequate response may result in poor oocyte production and maturation, and thus high cycle cancellation and low pregnancy rates. As multiple follicular growth is crucial, it remains a major challenge and a frustrating issue in this population of patients [21].

Although the concept of poor ovarian response (POR) was introduced more than 30 years ago, until 2011 we did not have a consensus on its definition. Polyzos and Devroey, in 2011, pointed out the enormous variability of definitions: in 47 randomized studies there were 41 different definitions [22]. These data not only confirmed the difficulty of estimating the prevalence of this condition (9%–24%, but perhaps slightly increasing lately) but also

highlighted how the results cannot be compared, as well as how variable are the strategies for its prevention and management. In the same year, an ESHRE working group on poor responders finally established in Bologna some criteria to define poor responders as women fulfilling at least two of the following characteristics:

- A previous episode of poor ovarian response (three or fewer oocytes) with a standard dose of drugs
- An abnormal ovarian reserve, with AFC <5–7 follicles or AMH <0.5–1.1 ng/mL
- Advanced maternal age (> 40 years)
- Previous ovarian surgery, genetic defects, chemotherapy, radiotherapy, and autoimmune diseases

The main purpose of the Bologna criteria was identifying a homogeneous population, yet they raised some criticisms. They represented a first step, but more precise risk factors were required, especially for young women [23].

A few years ago, another consensus document was released: the POSEIDON (Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number) classification [24] (Figure 54.1). It aimed at stratifying patients with low response after COS as follows:

- Group 1:* women aged <35 years with adequate ovarian reserve (AFC ≥5, AMH ≥1.2 ng/mL) with an unexpected poor response (fewer than four oocytes) or a suboptimal response (four to nine oocytes)
- Group 2:* women aged ≥35 years with adequate ovarian reserve (AFC ≥5, AMH ≥1.2 ng/mL) with an unexpected poor response (fewer than four oocytes) or a suboptimal response (four to nine oocytes)
- Group 3:* women aged <35 years with poor ovarian reserve (AFC <5, AMH <1.2 ng/mL)
- Group 4:* women aged ≥35 years with poor ovarian reserve (AFC <5, AMH <1.2 ng/mL).

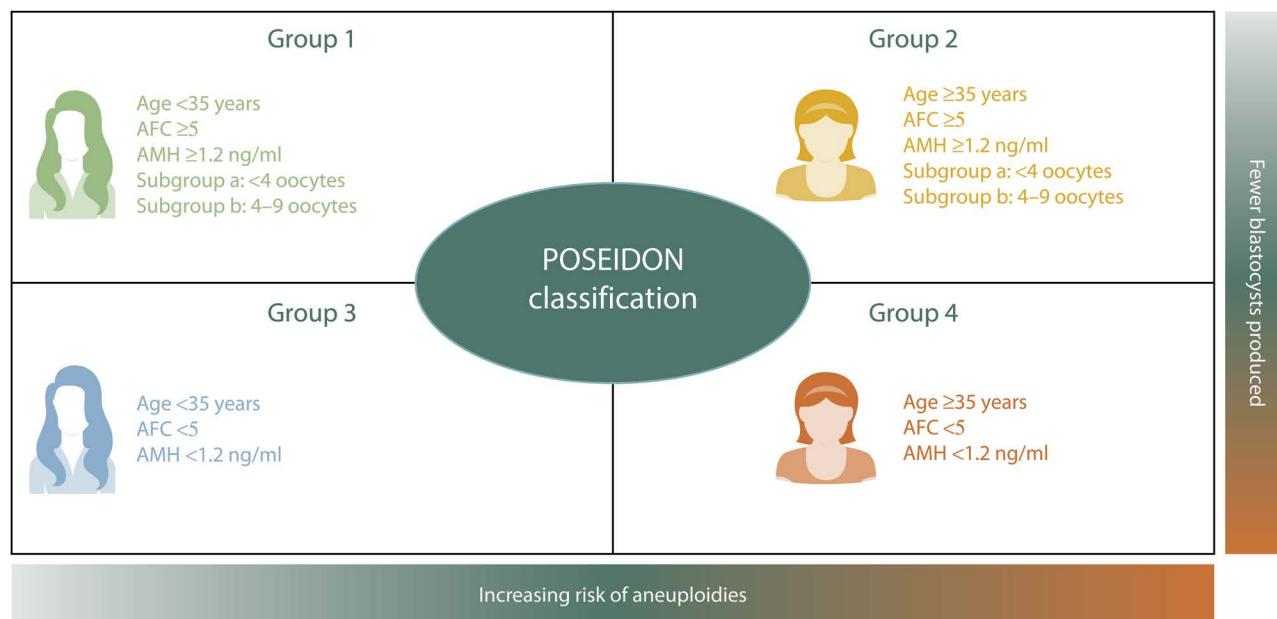


FIGURE 54.1 POSEIDON classification of poor prognosis patients. Four groups of poor-prognosis patients can be defined based on maternal age, ovarian reserve, previous response to controlled ovarian stimulation (COS), and expected aneuploidy rate at the blastocyst stage.

The POSEIDON classification allowed the definition of two main categories, “expected” (groups 3 and 4) and “unexpected” poor responders (groups 1 and 2). The POSEIDON criteria could help clinicians exploring patient-oriented strategies to retrieve the number of oocytes needed to obtain at least one euploid embryo in poor-prognosis IVF patients (Figure 54.1) [25]. Although over the last 20 years, many protocols with different doses and types of gonadotropins have been proposed to manage poor responders, to date there is still no truly effective treatment [5].

Several authors suggested GnRH antagonists in combination with gonadotropins as a suitable protocol. The use of GnRH antagonists in the mid-to-late follicular phase, during COS, prevents premature rise in LH surge, thereby achieving a more natural follicular recruitment, without the inhibitory effect possibly induced by the analogue.

In a recent meta-analysis of 14 randomized controlled trials, protocols with GnRH antagonists showed a shorter duration of stimulation than protocols with GnRH agonists, but no significant difference in the clinical outcome was reported. Since there is no ideal stimulation protocol that significantly improves the clinical outcome in poor responders, the use of GnRH antagonists could be considered the first-line treatment for COS [26]. One of the disadvantages of this protocol is that it relies on the AFC. Based on the AFC, it will be possible to decide whether starting COS or rather waiting for the following cycle, hoping for a higher AFC thanks to its inherent inter-cycle variability [27].

DuoStim: A novel strategy to manage poor responders

The improvement in cryopreservation protocols and the knowledge of the extreme dynamism of folliculogenesis has allowed the introduction of a new COS strategy entailing two stimulations in a single ovarian cycle (DuoStim) [3, 28]. Schematically, this protocol consists of a first COS followed by a first oocyte retrieval, a five-day pause, and a second COS followed by a second oocyte retrieval. In both cases, ovulation is triggered with a GnRH agonist (a single subcutaneous bolus of buserelin at a dose of 0.5 mL or triptorelin 0.3 mL) (Figure 54.2). The aim is

to reduce the half-life of the corpora lutei after egg retrieval and facilitate follicular recruitment from the second wave. Oocyte retrieval is performed 35 hours after the trigger in both COS. The rationale for this approach is to increase the number of oocytes collected from a single ovarian cycle. In a comparative study carried out on patients undergoing oocyte donation, Martinez et al. found no difference between follicular and luteal phase stimulations in terms of fertilization, implantation, and pregnancy rates [29]. Ubaldi et al. also showed that this protocol applied in 51 advanced maternal age and reduced ovarian reserve (five or fewer oocytes collected in the previous cycle; AMH \leq 1.5 ng/mL, AFC \leq 6 follicles) patients increased the chance of obtaining at least one euploid embryo from 42% to 65% in less than one month during PGT-A cycles [30]. In 2018, a case-control study was conducted by Cimadomo et al. where paired cohorts of oocytes collected from a first and a second COS in the same ovarian cycle were compared, confirming similar competence in terms of maturation, fertilization, blastulation, and euploidy rates, but also that on average the second cohort of oocytes was larger [31]. Vaiarelli et al. confirmed the reproducibility of DuoStim across 310 poor-prognosis patients from four IVF centres [32].

The higher number of oocytes collected after the second COS is imputable to the high level of oestradiol and progesterone after the first COS that may (i) better synchronize the cohort of antral follicles of the anovulatory wave; (ii) stimulate the proliferation of FSH receptors in their granulosa cells, thus leading to a better overall response to COS; (iii) change the ovarian micro-environment; (iv) increase angiogenic factors; (v) enhance the sensitivity of the granulosa cells to FSH; and/or (vi) elicit a flare-up effect from the triggering of the GnRH agonist which could induce a downregulation in AMH expression in anovulatory wave follicles. However, further studies are needed to confirm these assumptions [31, 33].

A SWOT analysis [32] (a tool to assess the strengths, weaknesses, opportunities, and threats of a project) in 2018 about DuoStim highlighted the absence of randomized studies to assess its (cost-) effectiveness compared to two conventional COSSs conducted in two consecutive ovarian cycles. Yet, its advantages as a strategy to reduce treatment discontinuation and increase the chance of success in a limited timeframe are concordant across

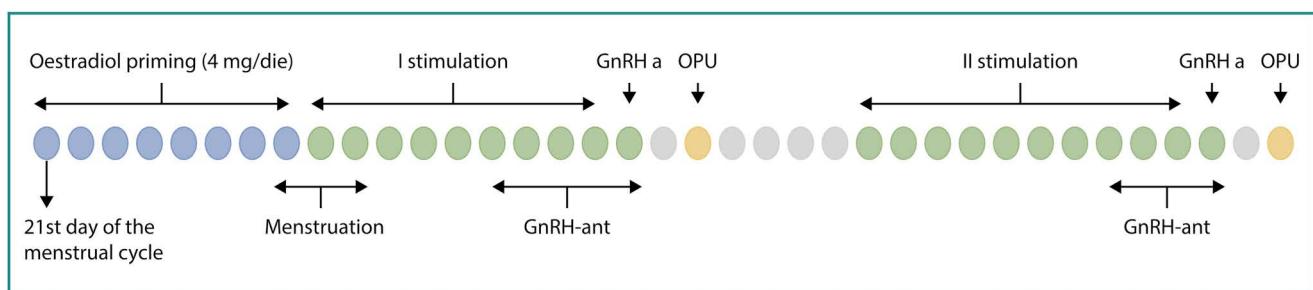


FIGURE 54.2 DuoStim protocol. Luteal oestradiol priming (4 mg/day of oestradiol valerate) is started on day 21 of the previous menstrual cycle to synchronize the follicular growth. After transvaginal ultrasound and basal ovarian assessment, on day 2 or 3 of the menstrual cycle, luteal oestradiol priming is stopped, and the first stimulation is started on day 2 of the menstrual cycle with a fixed 300 IU of recombinant FSH and 150 IU of recombinant LH for 4 days. Follicular growth is monitored with ultrasound scans on day 5, and then every 2–3 days. Daily administration of a GnRH antagonist (GnRH-ant) is started when a leading follicle with 13–14 mm diameter is identified and continued until the day of ovulation trigger. When at least two follicles reach 17–18 mm in diameter, ovulation is triggered with the agonist (GnRH a), and oocyte pick up (OPU) is performed 35 hours later. Five days after the first oocyte retrieval, a second stimulation in the same ovarian cycle is started with a GnRH-ant protocol identical to the first one.

all the studies published at present [34]. Also, patients fulfilling the Bologna criteria benefit from this protocol, doubling their CLBR per ITT (8% to 15%) when compared to the standard approach [35]. The most recently published case series showed that DuoStim can even be suggested in progress based on the embryological results of the first COS, and that this strategy is always more cost-effective than the conventional approach in terms of CLBR per ITT within one year at a willingness to pay threshold of 23,100 euros [36]. A strict follow-up is advisable for both patients and new-borns, but the data reported to date testify to the safety of this protocol in terms of embryological, clinical, and perinatal outcomes [37].

Framework of DuoStim

Luteal oestradiol priming (4 mg/day of oestradiol valerate) is started in day 21 of the previous menstrual cycle to promote the synchronization of follicular growth. After transvaginal ultrasound and basal ovarian assessment, on day 2 or 3 of the menstrual cycle, luteal oestradiol priming is stopped, and FPS is started with fixed dose of rec-FSH 300 IU/day plus rec-LH 75–150 IU/day for four days. Follicular growth is monitored on day 5 and then every two or three days. The GnRH antagonist is administered daily after identification of a leading follicle with a diameter ≥13–14 mm during both FPS and second stimulation until the day of ovulation trigger. Final oocyte maturation is triggered with a GnRH agonist to reduce the time of luteolysis. Egg retrieval is performed 35 hours after the trigger. Five days after the first retrieval, namely, the time needed to complete luteolysis, the second stimulation is started with the same protocol and the same daily dose regardless of the number of antral follicles detected by ultrasound scan in the anovulatory wave (Figure 54.2). A freeze-all approach must be adopted, and vitrified-warmed transfers performed in a modified natural or artificial cycle [32].

Open question: Is the second stimulation in the same ovarian cycle a real luteal phase stimulation?

Some authors recently suggested that “luteal phase stimulation” is improperly adopted to define the second COS performed after a conventional FPS in the context of DuoStim [38]. This is certainly an interesting comment that highlights the need for more studies on this topic. Specifically, although the adoption of the GnRH agonist trigger reduces the duration of the luteal phase, hormone levels during this period of the cycle are different from those found during a conventional COS. Moreover, the ovarian cycle is asynchronous to the endometrial cycle. Luteolysis is patient specific and highly dependent on hormone levels, the number of oocytes retrieved, and the number of corpora lutea, making the date of menstruation unpredictable. Secondly, in the absence of a second COS, follicle development at this stage of the ovarian cycle never reaches full maturity. In other words, this second stimulation makes it possible to collect those oocytes that would have grown and regressed in the luteal phase. Therefore, from Kuang’s publication onwards, the term “luteal phase stimulation” has been conventionally adopted [39]. Thirdly, data on pure luteal phase stimulations are very limited to date [40]. A study comparing pure-follicular versus pure-luteal phase stimulation conducted in the same patient in consecutive ovarian cycles has the potential to reveal important data soon. Up to that date, the terminology “luteal phase stimulation” will be considered improper and better we should better define it “second stimulation in the same ovarian cycle” [41]. An international consensus on the real

nature of the anovulatory waves exploited in the DuoStim protocol is certainly desirable.

Alternative approaches to manage poor responders

Mild stimulation

Mild ovarian stimulation is defined as a protocol in which the ovaries are stimulated with gonadotropins and/or other pharmacological compounds to allow the development of a limited number of follicles. The exact definition is variable. The conventional daily dose of FSH is 150–225 IU, while mild stimulation is achieved by using a lower dose of FSH, or a delayed start. Referring to the concept of “FSH window,” the administration of low-dose gonadotropins can be delayed until the mid to late follicular phase, supporting a more physiological recruitment and selection of the growing follicles. The proposed rationale is that competent oocytes are more probably contained within naturally selected follicles [42]. However, this theoretical qualitative advantage corresponds to a lower number of oocytes collected [43]. This results into a lower number of embryos obtained and, therefore, to a lower number of surplus embryos and a decreased CLBR. This protocol has been in fact challenged by a solid literature that shows that increasing chance of success corresponds to more oocytes collected from both a fresh ET and a cumulative perspective [6, 7, 44].

Two meta-analyses compared mild and conventional COS and reported no difference in pregnancy outcomes [45, 46], suggesting that two are equally effective for poor responders but the former is less expensive. However, none of them reported the CLBR per ITT or patient discontinuation rates.

In the era of personalized medicine, poor responders cannot be considered a single category of patient, and a “one-size-fits all” approach is therefore not feasible [47, 48].

Modified natural cycle

Modified natural cycle (MNC) is defined as a procedure in which one or more oocytes are collected from the ovaries during a spontaneous menstrual cycle. Drugs are administered with the sole purpose of blocking the spontaneous LH surge and/or inducing final oocyte maturation. In 2009, Schimberni et al. studied a series of 500 natural cycles in poor responders. The pregnancy rates per ET were encouraging: 29.2% in patients aged 35 years or less, 20.6% for women aged 36–39 years, and 10.5% in women aged 40 years or more [49]. When MNC in poor responders was compared to standard COS, the pregnancy rates per cycle and ET were not significantly different between treatment groups across different maternal age subclasses [50, 51]. It was then hypothesized that MNC is as effective as standard COS, with the benefit that gonadotropins are not needed. Certainly, one aspect in favour of the spontaneous cycle with or without minimal stimulation in poor responders and in case of mono-follicular growth, is the cost-benefit ratio compared to conventional COS. A retrospective study conducted by Drakopoulos et al. in advanced maternal age Bologna patients showed that MNC-IVF is a patient-friendly approach and a reasonable option in patients with low chances of recovering more than two oocytes [52]. Moreover, the use of MNC should be considered only in patients who require IVF with proven endocrinological evidence of ovarian ageing and in those who have had one or two previous cancelled COS attempts [53]. Indeed, MNC is probably not recommended over conventional COS as a first line treatment

for expected poor responders [5]. However MNC has a lower cost and with less discomfort for the patients and it is plausible to offer it as a second-line treatment for poor responders who do not respond to standard COS protocols [54].

Oestradiol priming in the luteal phase before ovarian stimulation with antagonist protocol

Different kinds of pre-treatment strategies have been proposed as adjuvant therapy prior to COS in poor responders. Among them, the use of oestradiol-based drugs (E2) administered during the luteal phase of the cycle prior to stimulation may improve the response, thereby reducing the risk of cycle cancellation in poor responders. Moreover, it has been shown that the administration of E2 orally, vaginally, or transdermally could help synchronize the pool of antral follicles available to grow in an antagonist protocol. In detail, this process is thought to be part of a negative feedback mechanism of the reproductive axis, acting on the inhibition of GnRH secretion and, consequently, gonadotropin response [55–57]. Despite this, a recent meta-analysis showed that the addition of E2 to the stimulation protocol by any route does not improve the efficacy of COS in terms of clinical pregnancy rate per patient [58–60].

The addition of LH activity during ovarian stimulation

The question is still open regarding the alleged benefit of additional LH activity during gonadotropin stimulation in poor responders. It has been suggested it may improve both response

to COS and IVF outcomes [61]. Especially in hypo-responders, several groups adopted hormones with LH activity, like recombinant LH (rec-LH), recombinant human chorionic gonadotrophin (rec-hCG), urinary hCG (u-hCG), and u-hCG contained in human chorionic menopausal gonadotrophin (HMG) [62–65]. Still, it is unclear whether any of them involves better outcomes than the others [66]. A recent review critically summarized the available evidence by comparing the effect of two commercially available LH preparations (i.e. HMG and rec-FSH + rec-LH) characterized by different sources of intrinsic LH bioactivity (i.e. hCG versus LH) on COS characteristics and IVF outcomes. No statistically significant difference was observed [67].

According to a Cochrane review, LH supplementation has no benefit on ongoing pregnancy [68] and, even in advanced maternal age women, the adjusted OR was 0.99 (CI 0.76–1.29) [69]. Conversely, a recent meta-analysis of 40 randomized controlled trials suggested that significantly more oocytes are recovered when both rec-FSH and rec-LH are used, in turn involving up to 30% higher clinical pregnancy rates in poor responders [70]. Another meta-analysis, then, supported that LH involves a better response even in advanced maternal age hypo-responders (fewer than nine follicles after COS) [65]. Yet, although some evidence of a potential benefit exists, it is insufficient to support that LH activity is beneficial in poor responder patients [71].

Addition of androgens

Some evidence showed that androgens may be key in early follicular development [72] by increasing the number of pre-antral

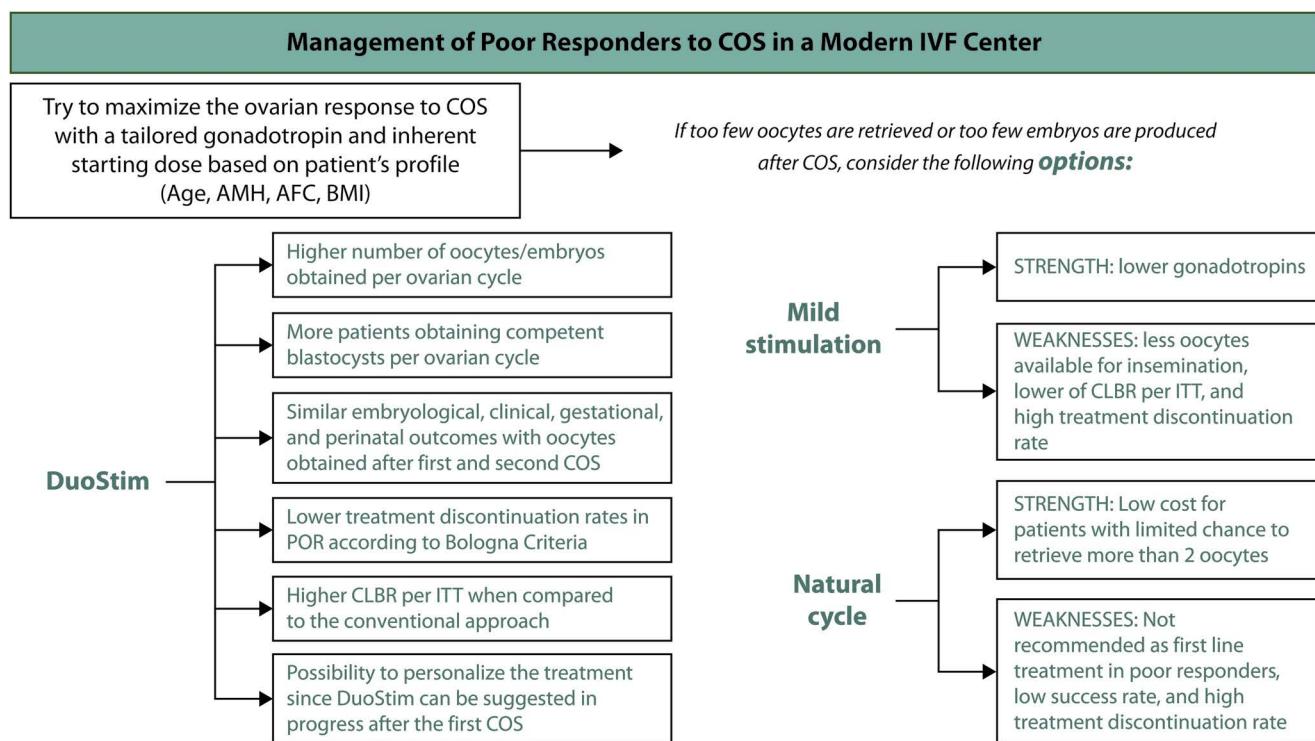


FIGURE 54.3 Summary of the main approaches to manage poor prognosis patients in a modern IVF centre. The first line approach is to adopt a tailored controlled ovarian stimulation (COS) strategy to attempt at maximizing the ovarian response, based on maternal age, AMH, antral follicle count (AFC), and body mass index (BMI). If too few oocytes are obtained or too few blastocysts are produced, the main options are DuoStim, mild stimulation, or natural cycle (in patients with limited chance to retrieve more than two oocytes). Abbreviations: CLBR, cumulative live birth rate; ITT, intention to treat.

and antral follicles and by acting on the proliferation of granulosa cells, which are the substrate for aromatic activity in the conversion of androgens into oestrogens. Furthermore, some authors have shown that androgens may increase the expression of FSH receptors in granulosa cells. However, the exact mechanisms of how androgens work are still under investigation. Transdermal testosterone is the most common route of administration, and some authors consider it a promising strategy with a solid biological rationale. However, there is currently inconsistent evidence that adjuvant testosterone pre-treatment before COS improves the number of oocytes retrieved and/or the clinical outcomes in poor responders undergoing IVF. Also, due to insufficient data on dosage, administration duration and safety, testosterone use cannot be recommended until a large randomized controlled trial will be conducted [5].

Conclusion

Advances in IVF, like blastocyst culture, aneuploidy testing, and oocyte/embryo cryopreservation profoundly changed the treatment of infertile couples, encouraging the clinicians to maximize the exploitation of the ovarian reserve through tailored protocols, especially for poor-prognosis patients. Concrete evidence suggests that, in this vitrification era, we must consider CLBR per ITT as our main outcome and aim at retrieving as many oocytes as possible after COS, especially in poor-prognosis patients. In this scenario, above all strategies suggested in poor responders (summarized in Figure 54.3), DuoStim figures amongst the most promising protocols; in fact, it allows maximizing the number of oocytes retrieved (and embryos produced) in the shortest possible timeframe, even in patients like the ones fulfilling the Bologna criteria. Currently, data from independent groups outlined the safety, consistency, and reproducibility of this approach, especially since it prevents the patients from discontinuing the treatment while still having a reasonably good chance to conceive with their own eggs. Currently, DuoStim can be suggested even in due course after conventional COS, making it a valuable strategy to increase patient centeredness and fully personalize IVF treatments. Clearly, an extensive counselling on the pros and cons of DuoStim is crucial, along with a strict follow-up of both patients and new-borns, the worldwide standardization of the protocol, prospective randomized studies, and cost-benefit analyses.

References

1. Ubaldi FM, Cimadomo D, Vaiarelli A, Fabozzi G, Venturella R, Maggiulli R, et al. Advanced maternal age in IVF: Still a challenge? The present and the future of its treatment. *Front Endocrinol (Lausanne)*. 2019;10:94.
2. Rienzi L, Cimadomo D, Vaiarelli A, Gennarelli G, Holte J, Livi C, et al. Measuring success in IVF is a complex multidisciplinary task: Time for a consensus? *Reprod Biomed Online*. 2021;43(5):775–8.
3. Venturella R, Vaiarelli A, Cimadomo D, Pedri S, Lico D, Mazzilli R, et al. State of the art and emerging drug therapies for female infertility. *Gynecol Endocrinol*. 2019;35(10):835–41.
4. Himabindu Y, Sriharibabu M, Gopinathan K, Satish U, Louis TF, Gopinath P. Anti-mullerian hormone and antral follicle count as predictors of ovarian response in assisted reproduction. *J Hum Reprod Sci*. 2013;6(1):27–31.
5. Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, Kolibianakis E, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI(dagger). *Hum Reprod Open*. 2020;2020(2):hoaa009.
6. Sunkara SK, Rittenberg V, Raine-Fenning N, Bhattacharya S, Zamora J, Coomarasamy A. Association between the number of eggs and live birth in IVF treatment: An Analysis of 400 135 treatment cycles. *Hum Reprod*. 2011;26(7):1768–74.
7. Polyzos NP, Drakopoulos P, Parra J, Pellicer A, Santos-Ribeiro S, Tournaye H, et al. Cumulative live birth rates according to the number of oocytes retrieved after the first ovarian stimulation for in vitro fertilization/intracytoplasmic sperm injection: A multicenter multinational analysis including approximately 15,000 women. *Fertil Steril*. 2018;110(4):661–70 e1.
8. Law YJ, Zhang N, Venetis CA, Chambers GM, Harris K. The number of oocytes associated with maximum cumulative live birth rates per aspiration depends on female age: A population study of 221 221 treatment cycles. *Hum Reprod*. 2019;34(9):1778–87.
9. Maggiulli R, Cimadomo D, Fabozzi G, Papini L, Dovere L, Ubaldi FM, et al. The effect of ICSI-related procedural timings and operators on the outcome. *Hum Reprod*. 2020;35(1):32–43.
10. Cimadomo D, Fabozzi G, Vaiarelli A, Ubaldi N, Ubaldi FM, Rienzi L. Impact of maternal age on oocyte and embryo competence. *Front Endocrinol (Lausanne)*. 2018;9:327.
11. Capalbo A, Hoffmann ER, Cimadomo D, Ubaldi FM, Rienzi L. Human female meiosis revised: New insights into the mechanisms of chromosome segregation and aneuploidies from advanced genomics and time-lapse imaging. *Hum Reprod Update*. 2017;23(6):706–22.
12. Franasiak JM, Forman EJ, Hong KH, Werner MD, Upham KM, Treff NR, et al. The nature of aneuploidy with increasing age of the female partner: A review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertil Steril*. 2014;101(3):656–63.e1.
13. Brown JB. Pituitary control of ovarian function—concepts derived from gonadotrophin therapy. *Aust N Z J Obstet Gynaecol*. 1978;18(1):46–54.
14. Findlay JK, Drummond AE, Dyson ML, Baillie AJ, Robertson DM, Ethier JF. Recruitment and development of The follicle; The roles of The transforming growth factor-beta superfamily. *Mol Cell Endocrinol*. 2002;191(1):35–43.
15. Filicori M, Cognigni GE, Samara A, Melappioni S, Perri T, Cantelli B, et al. The use of LH activity to drive folliculogenesis: Exploring uncharted territories in ovulation induction. *Hum Reprod Update*. 2002;8(6):543–57.
16. Vaiarelli A, Venturella R, Vizziello D, Bulletti F, Ubaldi FM. Dual ovarian stimulation and random start in assisted reproductive technologies: From ovarian biology to clinical application. *Curr Opin Obstet Gynecol*. 2017;29(3):153–9.
17. Baerwald AR, Adams GP, Pierson RA. Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Hum Reprod Update*. 2012;18(1):73–91.
18. Hughes EG, Fedorkow DM, Daya S, Sagle MA, Van de Koppel P, Collins JA. The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: A meta-analysis of randomized controlled trials. *Fertil Steril*. 1992;58(5):888–96.
19. Daya S. Gonadotropin releasing hormone agonist protocols for pituitary desensitization in in vitro fertilization and gamete intrafallopian transfer cycles. *Cochrane Database Syst Rev*. 2000(2):CD001299.
20. Olivennes F, Cunha-Filho JS, Fanchin R, Bouchard P, Frydman R. The use of GnRH antagonists in ovarian stimulation. *Hum Reprod Update*. 2002;8(3):279–90.
21. Vaiarelli A, Cimadomo D, Ubaldi N, Rienzi L, Ubaldi FM. What is new in the management of poor ovarian response in IVF? *Curr Opin Obstet Gynecol*. 2018;30(3):155–62.
22. Polyzos NP, Devroey P. A systematic review of randomized trials for the treatment of poor ovarian responders: Is there any light at the end of the tunnel? *Fertil Steril*. 2011;96(5):1058–61.e7.

23. Drakopoulos P, Bardhi E, Boudry L, Vaiarelli A, Makrigiannakis A, Esteves SC, et al. Update on The management of poor ovarian response in IVF: The shift from Bologna criteria to The poseidon concept. *Ther Adv Reprod Health.* 2020;14:2633494120941480.
24. Alviggi C, Andersen CY, Buehler K, Conforti A, De Placido G, Esteves SC, et al. Poseidon Group. A new more detailed stratification of low responders to ovarian stimulation: From a poor ovarian response to a low prognosis concept. *Fertil Steril.* 2016;105(6):1452–3.
25. Conforti A, Esteves SC, Cimadomo D, Vaiarelli A, Rella D, Ubaldi F, et al. Management of women with an unexpected low ovarian response to gonadotropin. *Front Endocrinol (Lausanne).* 2019;10:387.
26. Patrizio P, Vaiarelli A, Levi Setti PE, Tobler KJ, Shoham G, Leong M, et al. How to define, diagnose and treat poor responders? Responses from a worldwide survey of IVF clinics. *Reprod Biomed Online.* 2015;30(6):581–92.
27. Ubaldi F, Vaiarelli A, D'Anna R, Rienzi L. Management of poor responders in IVF: Is there anything new? *Biomed Res Int.* 2014;2014:352098.
28. Pailis M, Sapir O, Lande Y, Ben-Haroush A, Altman E, Wertheimer A, et al. Consecutive ovarian stimulation is beneficial in patients with a poor response to high-dose follicle-stimulating hormone. *Gynecol Endocrinol.* 2021;37(11):995–9.
29. Martinez F, Clua E, Devesa M, Rodriguez I, Arroyo G, Gonzalez C, et al. Comparison of starting ovarian stimulation on day 2 versus day 15 of the menstrual cycle in the same oocyte donor and pregnancy rates among the corresponding recipients of vitrified oocytes. *Fertil Steril.* 2014;102(5):1307–11.
30. Ubaldi FM, Capalbo A, Vaiarelli A, Cimadomo D, Colamaria S, Alviggi C, et al. Follicular versus luteal phase ovarian stimulation during the same menstrual cycle (DuoStim) in a reduced ovarian reserve population results in a similar euploid blastocyst formation rate: New insight in ovarian reserve exploitation. *Fertil Steril.* 2016;105(6):1488–95.e1.
31. Cimadomo D, Vaiarelli A, Colamaria S, Trabucco E, Alviggi C, Venturella R, et al. Luteal phase anovulatory follicles result in the production of competent oocytes: Intra-patient paired case-control study comparing follicular versus luteal phase stimulations in the same ovarian cycle. *Hum Reprod.* 2018;33(8):1442–8.
32. Vaiarelli A, Cimadomo D, Trabucco E, Vallefouco R, Buffo L, Dusi L, et al. Double stimulation in the Same ovarian cycle (DuoStim) to maximize the number of oocytes retrieved from poor prognosis patients: A multicenter experience and SWOT analysis. *Front Endocrinol (Lausanne).* 2018;9:317.
33. Vaiarelli A, Cimadomo D, Argento C, Ubaldi N, Trabucco E, Drakopoulos P, et al. Double stimulation in the same ovarian cycle (DuoStim) is an intriguing strategy to improve oocyte yield and the number of competent embryos in a short timeframe. *Minerva Ginecol.* 2019;71(5):372–6.
34. Vaiarelli A, Cimadomo D, Petriglia C, Conforti A, Alviggi C, Ubaldi N, et al. DuoStim - a reproducible strategy to obtain more oocytes and competent embryos in a short time-frame aimed at fertility preservation and IVF purposes. A systematic review. *Ups J Med Sci.* 2020;125:1–10.
35. Vaiarelli A, Cimadomo D, Conforti A, Schimberni M, Giuliani M, D'Alessandro P, et al. Luteal phase after conventional stimulation in the same ovarian cycle might improve the management of poor responder patients fulfilling the Bologna criteria: A case series. *Fertil Steril.* 2020;113(1):121–30.
36. Vaiarelli A, Cimadomo D, Gennarelli G, Guido M, Alviggi C, Conforti A, et al. Second stimulation in the same ovarian cycle: An option to fully-personalize the treatment in poor prognosis patients undergoing PGT-A. *J Assist Reprod Genet.* 2022;39(3):663–73.
37. Vaiarelli A, Cimadomo D, Alviggi E, Sansone A, Trabucco E, Dusi L, et al. The euploid blastocysts obtained after luteal phase stimulation show the same clinical, obstetric and perinatal outcomes as follicular phase stimulation-derived ones: A multicenter study. *Hum Reprod.* 2020;35(11):2598–608.
38. Racca A, Polyzos NP. DuoStim: Are we really comparing follicular phase with luteal phase stimulations? *Hum Reprod.* 2021;36(6):1722–3.
39. Kuang Y, Chen Q, Hong Q, Lyu Q, Ai A, Fu Y, et al. Double stimulations during the follicular and luteal phases of poor responders in IVF/ICSI programmes (Shanghai protocol). *Reprod Biomed Online.* 2014;29(6):684–91.
40. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: Systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update.* 2017;23(2):139–55.
41. Vaiarelli A, Cimadomo D, Rienzi L, Ubaldi FM. Reply: 'Second stimulation in the same ovarian cycle', probably a terminology more appropriate than 'luteal phase stimulation' in the DuoStim protocol. *Hum Reprod.* 2021;36(6):1723–4.
42. Baart EB, Martini E, Eijkemans MJ, Van Opstal D, Beckers NG, Verhoeff A, et al. Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: A randomized controlled trial. *Hum Reprod.* 2007;22(4):980–8.
43. Siristatidis C, Salamalekis G, Dafopoulos K, Basios G, Vogiatzi P, Papantoniou N. Mild versus conventional ovarian stimulation for poor responders undergoing IVF/ICSI. *In Vivo.* 2017;31(2):231–7.
44. Drakopoulos P, Blockeel C, Stoop D, Camus M, de Vos M, Tournaye H, et al. Conventional ovarian stimulation and single embryo transfer for IVF/ICSI. How many oocytes do we need to maximize cumulative live birth rates after utilization of all fresh and frozen embryos? *Hum Reprod.* 2016;31(2):370–6.
45. Datta AK, Maheshwari A, Felix N, Campbell S, Nargund G. Mild versus conventional ovarian stimulation for IVF in poor, normal and hyper-responders: A systematic review and meta-analysis. *Hum Reprod Update.* 2021;27(2):229–53.
46. Datta AK, Maheshwari A, Felix N, Campbell S, Nargund G. Mild versus conventional ovarian stimulation for IVF in poor responders: A systematic review and meta-analysis. *Reprod Biomed Online.* 2020;41(2):225–38.
47. Polyzos NP, Popovic-Todorovic B. SAY NO to mild ovarian stimulation for all poor responders: It is time to realize that not all poor responders are the same. *Hum Reprod.* 2020;35(9):1964–71.
48. Romito A, Bardhi E, Errazuriz J, Blockeel C, Santos-Ribeiro S, Vos M, et al. Heterogeneity among poor ovarian responders according to Bologna criteria results in diverging cumulative live birth rates. *Front Endocrinol (Lausanne).* 2020;11:208.
49. Schimberni M, Morgia F, Colabianchi J, Giallonardo A, Piscitelli C, Giannini P, et al. Natural-cycle in vitro fertilization in poor responder patients: A survey of 500 consecutive cycles. *Fertil Steril.* 2009;92(4):1297–301.
50. Morgia F, Sbracia M, Schimberni M, Giallonardo A, Piscitelli C, Giannini P, et al. A controlled trial of natural cycle versus microdose gonadotropin-releasing hormone analog flare cycles in poor responders undergoing in vitro fertilization. *Fertil Steril.* 2004;81(6):1542–7.
51. Kim CH, Kim SR, Cheon YP, Kim SH, Chae HD, Kang BM. Minimal stimulation using gonadotropin-releasing hormone (GnRH) antagonist and recombinant human follicle-stimulating hormone versus GnRH antagonist multiple-dose protocol in low responders undergoing in vitro fertilization/intracytoplasmic sperm injection. *Fertil Steril.* 2009;92(6):2082–4.
52. Drakopoulos P, Romito A, Errazuriz J, Santos-Ribeiro S, Popovic-Todorovic B, Racca A, et al. Modified natural cycle IVF versus conventional stimulation in advanced-age Bologna poor responders. *Reprod Biomed Online.* 2019;39(4):698–703.
53. Kadoch IJ, Phillips SJ, Bissonnette F. Modified natural-cycle in vitro fertilization should be considered as the first approach in young poor responders. *Fertil Steril.* 2011;96(5):1066–8.
54. Ho JR, Paulson RJ. Modified natural cycle in in vitro fertilization. *Fertil Steril.* 2017;108(4):572–6.

55. Reynolds KA, Omurtag KR, Jimenez PT, Rhee JS, Tuuli MG, Jungheim ES. Cycle cancellation and pregnancy after luteal estradiol priming in women defined as poor responders: A systematic review and meta-analysis. *Hum Reprod.* 2013;28(11):2981–9.
56. Fanchin R, Salomon L, Castelo-Branco A, Olivennes F, Frydman N, Frydman R. Luteal estradiol pre-treatment coordinates follicular growth during controlled ovarian hyperstimulation with GnRH antagonists. *Hum Reprod.* 2003;18(12):2698–703.
57. Chang EM, Han JE, Won HJ, Kim YS, Yoon TK, Lee WS. Effect of estrogen priming through luteal phase and stimulation phase in poor responders in in-vitro fertilization. *J Assist Reprod Genet.* 2012;29(3):225–30.
58. Wu H, Zhang S, Lin X, Wang S, Zhou P. Luteal phase support for in vitro fertilization/intracytoplasmic sperm injection fresh cycles: A systematic review and network meta-analysis. *Reprod Biol Endocrinol.* 2021;19(1):103.
59. Farquhar C, Rombauts L, Kremer JA, Lethaby A, Ayeleke RO. Oral Contraceptive pill, progestogen or oestrogen pretreatment for ovarian stimulation protocols for women undergoing assisted reproductive techniques. *Cochrane Database Syst Rev.* 2017;5:CD006109.
60. Gelbaya TA, Kyrgiou M, Tsoumpou I, Nardo LG. The use of estradiol for luteal phase support in in vitro fertilization/intracytoplasmic sperm injection cycles: A systematic review and meta-analysis. *Fertil Steril.* 2008;90(6):2116–25.
61. van Wely M, Westergaard LG, Bossuyt PM, van der Veen F. Effectiveness of human menopausal gonadotropin versus recombinant follicle-stimulating hormone for controlled ovarian hyperstimulation in assisted reproductive cycles: A meta-analysis. *Fertil Steril.* 2003;80(5):1086–93.
62. Ferraretti AP, Gianaroli L, Magli MC, D'Angelo A, Farfalli V, Montanaro N. Exogenous luteinizing hormone in controlled ovarian hyperstimulation for assisted reproduction techniques. *Fertil Steril.* 2004;82(6):1521–6.
63. De Placido G, Alviggi C, Perino A, Strina I, Lisi F, Fasolino A, et al. Recombinant human LH supplementation versus recombinant human FSH (rFSH) step-up protocol during controlled ovarian stimulation in normogonadotropic women with initial inadequate ovarian response to rFSH. A multicentre, prospective, randomized controlled trial. *Hum Reprod.* 2005;20(2):390–6.
64. Alviggi C, Pettersson K, Longobardi S, Andersen CY, Conforti A, De Rosa P, et al. A common polymorphic allele of the LH beta-subunit gene is associated with higher exogenous FSH consumption during controlled ovarian stimulation for assisted reproductive technology. *Reprod Biol Endocrinol.* 2013;11:51.
65. Lehert P, Kolibianakis EM, Venetis CA, Schertz J, Saunders H, Arriagada P, et al. Recombinant human follicle-stimulating hormone (r-hFSH) plus recombinant luteinizing hormone versus r-hFSH alone for ovarian stimulation during assisted reproductive technology: Systematic review and meta-analysis. *Reprod Biol Endocrinol.* 2014;12:17.
66. Mak SM, Wong WY, Chung HS, Chung PW, Kong GW, Li TC, et al. Effect of mid-follicular phase recombinant LH versus urinary HCG supplementation in poor ovarian responders undergoing IVF - a prospective double-blinded randomized study. *Reprod Biomed Online.* 2017;34(3):258–66.
67. Orvieto R. HMG versus recombinant FSH plus recombinant LH in ovarian stimulation for IVF: Does the source of LH preparation matter? *Reprod Biomed Online.* 2019;39(6):1001–6.
68. Mochtar MH, Van der V, Ziech M, van Wely M. Recombinant luteinizing hormone (rLH) for controlled ovarian hyperstimulation in assisted reproductive cycles. *Cochrane Database Syst Rev.* 2007(2):CD005070.
69. Konig TE, van der Houwen LE, Overbeek A, Hendriks ML, Beutler-Beemsterboer SN, Kuchenbecker WK, et al. Recombinant LH supplementation to a standard GnRH antagonist protocol in women of 35 years or older undergoing IVF/ICSI: A randomized controlled multicentre study. *Hum Reprod.* 2013;28(10):2804–12.
70. Conforti A, Esteves SC, Humaidan P, Longobardi S, D'Hooghe T, Orvieto R, et al. Recombinant human luteinizing hormone co-treatment in ovarian stimulation for assisted reproductive technology in women of advanced reproductive age: A systematic review and meta-analysis of randomized controlled trials. *Reprod Biol Endocrinol.* 2021;19(1):91.
71. Mochtar MH, Danhof NA, Ayeleke RO, Van der Veen F, van Wely M. Recombinant luteinizing hormone (rLH) and recombinant follicle stimulating hormone (rFSH) for ovarian stimulation in IVF/ICSI cycles. *Cochrane Database Syst Rev.* 2017;5:CD005070.
72. Noventa M, Vitagliano A, Andrisani A, Blaganje M, Vigano P, Papaelo E, et al. Testosterone therapy for women with poor ovarian response undergoing IVF: A meta-analysis of randomized controlled trials. *J Assist Reprod Genet.* 2019;36(4):673–83.

ADJUVANTS FOR POOR RESPONDERS

Christos A. Venetis

Introduction

One of the key determinants of success in assisted reproductive technologies (ART) is the outcome of ovarian stimulation, which is used to induce multi-follicular growth. The goal is the retrieval of multiple oocytes, as it has been shown that the probability of pregnancy after stimulated ART cycles is higher compared to natural/unstimulated cycles [1]. Moreover, there have been several, predominantly observational, studies which have suggested that the probability of pregnancy or live birth after ART increases with a higher number of oocytes obtained [2].

This has created the clinical notion of an optimal number of oocytes retrieved which can lead to maximal pregnancy rates while at the same time keep the risk of ovarian hyperstimulation syndrome (OHSS) low [2, 3]. According to a recent systematic review of the literature, this number depends on the outcome measure used (e.g. achievement of pregnancy after a fresh embryo transfer vs cumulative pregnancy rate), as well as on the age of the woman undergoing ART. This has led to the categorization of patients and their response to ovarian stimulation to three main groups: (i) poor responders, who have a lower number of oocytes retrieved and therefore lower chance of a pregnancy, (ii) normal responders, with an optimal number of oocytes retrieved which achieves good pregnancy rates and minimizes the risk of OHSS, and (iii) hyper-responders, those who have an excessive number of oocytes retrieved and are at high risk for OHSS.

The therapeutic challenge of poor ovarian response

Poor ovarian response is a clinical entity which has been identified since the beginning of the ovarian stimulation era [4] and represents one of the most important therapeutic challenges, even in modern ART [5]. Poor responders have a higher risk of having their oocyte retrieval cancelled or obtaining a low number of oocytes, which increases the probability of fertilization failure or can, more commonly, lead to cancellation of the embryo transfer due to lack of suitable embryos for transfer.

The incidence of poor ovarian response has been reported to vary widely, ranging between 9% and 24% [6] and its proposed aetiology includes advanced age [7], previous ovarian surgery [8], pelvic adhesions [9], previous gonadotoxic treatment (chemotherapy or radiation) [10], and high body mass index [11], but it can also occur unexpectedly, mostly associated with premature ovarian insufficiency due to genetic factors, known or unknown [12, 13].

The challenges in the definition of poor ovarian response and interpreting the literature

Not unexpectedly, over the last four decades, a significant amount of research has been focused on improving the management of patients with poor ovarian response, with several interventions having been proposed and tested [14, 15], both in randomized and non-randomized clinical trials.

The most significant challenge in interpreting the literature, however, is the lack of uniformity in the definition of poor ovarian response. In a systematic review of 47 randomized controlled trials (RCTs), it was shown that 41 different definitions of poor ovarian response had been used [16]. The lack of a universally accepted definition of poor ovarian response leads to substantial clinical heterogeneity between published studies, which renders the synthesis of available evidence problematic. In an attempt to resolve this issue, the European Society of Human Reproduction and Embryology (ESHRE) issued a consensus paper in 2011, introducing the “Bologna criteria” for the definition of poor ovarian response [17]. These criteria defined poor ovarian response as follows:

At least two of the following three features must be present:

1. Advanced maternal age (≥ 40 years) or any other risk factor for poor ovarian response
2. A previous poor ovarian response (three or fewer oocytes with a conventional stimulation protocol)
3. An abnormal ovarian reserve test (i.e. antral follicle count (AFC): 5–7 follicles, or anti-Müllerian hormone (AMH): 0.5–1.1 ng/mL).

Two episodes of poor ovarian response after maximal stimulation are sufficient to define a patient as a poor responder in the absence of advanced maternal age or abnormal ovarian reserve test.

The introduction of these criteria has generally been welcomed as it provides the framework that would allow further studies to be performed on a relatively homogenous population. Nevertheless, it has been suggested that these criteria are too broad and can include populations with entirely different prognosis [18]. Although this is a possibility, the potential benefits from the adoption of the Bologna criteria, far outweigh the potential risks [19, 20].

Another step in defining poor ovarian response and low prognosis in a clinically meaningful way has been taken more recently with the introduction of the POSEIDON classification [21]. These criteria stratify patients based on their ovarian reserve markers and their age, constructing four different groups. Group 1 includes patients <35 years of age with adequate ovarian reserve parameters (AFC ≥ 5 ; AMH ≥ 1.2 ng/mL) and with an unexpected poor (fewer than four oocytes) or suboptimal (four to nine oocytes) response. Group 2 includes similar patients to group 1 but ≥ 35 years of age. Group 3 includes patients <35 years of age with poor ovarian reserve pre-stimulation parameters (AFC <5 ; AMH <1.2 ng/mL), and group 4 includes patients ≥ 35 years of age with the same poor ovarian reserve pre-stimulation parameters (AFC <5 ; AMH <1.2 ng/mL). It becomes apparent that groups 1 and 2 include patients with unexpected poor ovarian response after standard ovarian stimulation compared to groups 3 and 4, which include patients with expected poor response.

Strategies to treat poor ovarian response after ovarian stimulation for ART

In theory, the strategies to manage poor ovarian response after ovarian stimulation for ART could be grouped into three main categories: (i) to increase the number of recruitable follicles before the initiation of ovarian stimulation, (ii) to ensure that you recruit all available follicles during ovarian stimulation, and (iii) to increase the quality of the oocytes (Figure 55.1).

The first two strategies seem to focus more on addressing the issue of low ovarian reserve and response, respectively, whereas the third strategy aims to increase the probability of pregnancy in these patients by enhancing the quality of the collected oocytes and therefore their potential to produce competent embryos that can lead to a healthy pregnancy.

Based on these theoretical concepts, several different therapeutic interventions for the management of poor ovarian response have been proposed.

Many of these interventions are classified as “adjutants” or “add-ons” and their use during ovarian stimulation has been a matter of controversy. The Human Fertilisation & Embryology Authority in the United Kingdom defines treatment add-ons as optional, additional treatments (frequently involving additional cost for the patient) which often claim to be effective at improving the chances of having a baby (live birth rate) but the evidence to support this for most fertility patients is usually missing or not very reliable [22]. Despite the apparent lack of a solid evidence base, a recent survey in Australia and New Zealand involving 1590 women showed that 8 of 10 women had at some point during their treatment used one or more of these “add-ons” [23].

In a recent relevant discussion including experts and patient representatives organized by ESHRE it was agreed that the use of experimental treatments in assisted reproduction technology should ideally be performed within the context of well-designed clinical research, and at the bare minimum after the patient has been properly informed on the cost of such a treatment, its experimental nature, and, most importantly, on the available evidence around its efficacy and safety [24].

In the following section, the available evidence around the therapeutic value of the most common adjutants for poor ovarian response will be presented and discussed.

Androgens

The role of androgens in folliculogenesis

Human folliculogenesis involves a series of complex interactions between different types of cells that have distinct roles and work in synergy to achieve the growth and maturation of the follicle from the primordial to the pre-ovulatory stage. Accumulation of androgens in the micro-environment of the primate ovary has been suggested to play an important role in early follicular development and granulosa cell proliferation [25]. Androgen excess leads to an increase in the expression of the insulin-like growth factor I (IGF-I), can promote early stages of follicular growth [25–27], and increase the number of preantral and antral follicles [25, 28, 29]. In addition, increased intraovarian concentration of androgens seems to augment follicle stimulating hormone (FSH) receptor expression in granulosa cells [25, 28, 30] and, thus, potentially lead to enhanced responsiveness of ovaries to FSH [31, 32].

Moreover, clinical evidence from women with polycystic ovary syndrome or testosterone-treated female-to-male transsexuals, demonstrate that exposure to exogenous androgens may lead to increased number of developing follicles [33–35]. Finally, sub-optimal concentrations of endogenous androgens are associated with decreased sensitivity to ovarian stimulation with FSH and lower pregnancy rates after ART [36].

These experimental and clinical data combined have led to the hypothesis that increasing androgen concentration in the ovarian micro-environment in poor responders might result in an increase in the number and maturity of oocytes after ovarian stimulation for ART.

Hence, pre-treatment with various androgens has been proposed and tested [37, 38] in this special population, with the most researched being pre-treatment with transdermal testosterone [39] and with dehydroepiandrosterone (DHEA) [40].

Testosterone

Physiological basis of the intervention

Testosterone (T) along with its metabolite 5 α -dihydrotestosterone (DHT) represent two of the most potent androgens produced in

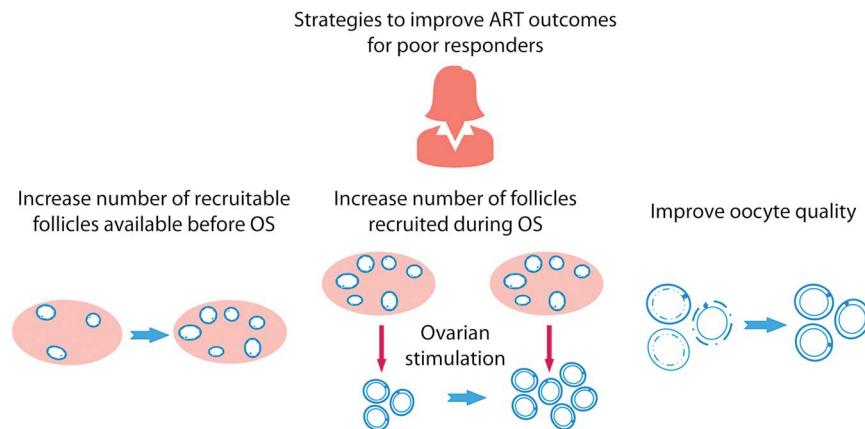


FIGURE 55.1 Strategies to treat poor ovarian response after ovarian stimulation for ART.

humans, and in women are mainly produced in the adrenal gland (25%), the ovaries (25%), and the peripheral compartment (50%). Most of the testosterone (~66%) is bound by sex hormone binding globulin (SHBG), and around 30% is bound by albumin, leaving only about 2% of testosterone free.

Importantly, there is a body of experimental evidence to suggest that DHT is associated with increased preantral follicle growth, increased FSH receptor mRNA and protein expression, reduced apoptosis of follicles, increased granulosa cell proliferation, and expression of steroidogenic enzymes [41]. This clearly showcases the potential of testosterone administration (the main precursor of DHT) and explains on why it has been used for the treatment of poor responders.

The main formulation of testosterone treatment has been transdermal gel as this has been shown to result in the most physiological increases of serum testosterone concentration. The percutaneous absorption of testosterone ranges between 9% and 14% of the administered dose. Based on pharmacokinetic studies performed in postmenopausal women, the serum testosterone concentration increases within a few hours after administration, providing relatively stable levels [42, 43], as it avoids the first-pass metabolism and the increased hepatic concentrations associated with the oral administration of testosterone.

Clinical evidence

Balasch et al. [39] were the first researchers to conduct a study on the potential value of transdermal testosterone pre-treatment for patients who had exhibited poor ovarian response in previous cycles. This was a prospective study, using self-controls, which evaluated the use of testosterone pre-treatment in 25 consecutive patients who had previously two cancelled IVF cycles due to poor follicular response despite having adequate stimulation with exogenous FSH. During the index cycle, patients were treated with a long, mid-luteal gonadotropin releasing hormone (GnRH) agonist protocol. As soon as pituitary suppression was confirmed, patients received transdermal testosterone using a patch aiming for a dose of 20 µg/kg of testosterone per day for five days before the initiation of ovarian stimulation with FSH. Compared to their first cancelled cycle, patients exhibited a five-fold increase in the number of recruited follicles, 80% of them underwent oocyte retrieval and produced on average 5.8 oocytes. All patients with an oocyte retrieval had an embryo transfer and 30% of them achieved a clinical pregnancy.

Following these very encouraging early data, several RCTs were performed. Massin et al. [44], in a double blind RCT, included 53 women (<42 years old) with previous poor response (defined as plasma oestradiol <1200 pg/mL on the day of human chorionic gonadotropin (hCG) administration and five or fewer oocytes retrieved) and evidence of a decreased ovarian reserve (determined at day 3 of a spontaneous cycle and defined as plasma hormonal values (FSH, E2, or inhibin B) outside the normal range of the local standard, i.e. FSH >12 IU/L, E2 <70 pg/mL, and inhibin B <45 pg/mL). They randomized 26 women to receive placebo and 27 women to receive 10 mg/d of transdermal testosterone (testosterone gel 1%) for 21 days prior to the initiation of ovarian stimulation. Women who were treated with a long GnRH agonist protocol had the testosterone pre-treatment during pituitary downregulation, and women treated with a GnRH antagonist protocol had transdermal testosterone during a cycle of OCP pre-treatment. Although an increase in the number of oocytes retrieved compared to their previous poor response cycle was observed in both the placebo and the

treatment group (likely due to a “regression to the mean” effect), there was no significant difference in the number of oocytes retrieved between the placebo (mean: 5.00 oocytes) and the transdermal testosterone group (mean: 5.31 oocytes). Similarly, there were no significant differences in the number of embryos produced and clinical pregnancy rates between the two groups compared [44].

The next RCT was performed by Kim et al. [45] who included 110 poor responders defined as women who had failed to produce three or more follicles with a mean diameter of ≥16 mm and resulted in three or fewer oocytes retrieved despite the use of a high gonadotropin dose (>2500 IU) in a previous failed IVF/ICSI cycle. All patients received oestrogen/progesterone pre-treatment for 21 days prior to ovarian stimulation. These patients were randomized to the transdermal testosterone gel pre-treatment group (n = 55) or the control group (n = 55). Patients in the pre-treatment group were administered 12.5 mg of transdermal testosterone gel (testosterone gel 1%). Significantly more oocytes were retrieved in the testosterone pre-treatment group (5.4 vs 3.8 oocytes retrieved; p <0.001). Clinical pregnancy rates per randomized patient were significantly increased in the testosterone pre-treatment group compared to the control group (30.9% vs 14.5%, respectively; p = 0.041). Live birth rates per randomized patient were higher, although not significantly so, in the testosterone pre-treatment group (27.3% vs 12.7%, respectively; p = 0.057).

The same group performed another RCT [46] on the same population where they tested the effect of testosterone pre-treatment on IVF outcomes depending on the duration of this pre-treatment. In brief, 30 patients were randomized in two, three, or four weeks of pre-treatment with testosterone gel (1%, 12.5 mg/day) and 30 patients were randomized to no pre-treatment and served as controls (N = 120, in total). The authors observed that the antral follicle count and the ovarian blood flow was increased in women who had received three or four weeks of pre-treatment compared to the control group. Similarly, lower total dose of gonadotropins was required to complete ovarian stimulation in the groups which had three or four weeks of pre-treatment with testosterone gel. Live birth rates per randomized patient were increased with the duration of testosterone gel pre-treatment (control: 6.7%, two weeks pre-treatment: 13.4%, three weeks pre-treatment: 20.0%, four weeks pre-treatment: 30.0%) but a statistically significant difference was present only when comparing four weeks of pre-treatment with the control group (p = 0.042). Overall, this was one of the first studies providing evidence that longer duration of testosterone pre-treatment might be required to observe an actual clinical benefit.

The first study to investigate the effect of transdermal testosterone in poor responders satisfying the Bologna criteria was the one by Bosdou et al. [47], which randomized 25 women in receiving pre-treatment with 10 mg of testosterone gel (2%) for 21 days and 25 women not receiving any pre-treatment. All women underwent a long follicular GnRH agonist protocol, and the primary outcome measure was the number of oocytes retrieved. No significant difference in the number of oocytes retrieved and live birth rates per randomized patient was found between the two groups compared in this study.

The next study to investigate the effect of transdermal testosterone in poor responders satisfying the Bologna criteria also randomized 25 women in receiving pre-treatment with testosterone gel or placebo gel from the second day of the menstrual cycle during a GnRH antagonist cycle [48]. The number of oocytes

retrieved was significantly higher in the testosterone pre-treatment group compared to the placebo group (testosterone: 2.48 oocytes vs placebo: 1.17 oocytes; $p = 0.004$). Similarly, clinical pregnancy rates were reported to be higher in the testosterone pre-treatment group compared to the placebo one (odds ratio-OR: 1.20, 95% CI: 1.01–1.43).

In 2021, Hoang et al. [49] performed a RCT in 159 poor responders according to the Bologna criteria, examining the therapeutic effect of prolonged testosterone pre-treatment. Specifically, 53 women were randomized to receive 12.5 mg of transdermal testosterone gel 1% for four weeks before commencing ovarian stimulation, 53 women were to receive 12.5 mg of transdermal testosterone gel 1% for six weeks, and 53 women were randomized to receive nothing. All patients underwent ovarian stimulation using a GnRH antagonist protocol and recombinant follitropin. Women who received four or six weeks of testosterone pre-treatment had shorter duration of stimulation and lower total dose of rFSH compared to the control group. No significant differences were observed in the number of oocytes retrieved between groups (5.5 vs 5.4 and 5.6 in control, the four-week or six-week group, respectively). Ongoing pregnancy rates were higher in the four-week (30%) or six-week (21.6%) group compared to the control group (7.5%), but this reached statistical

significance only when comparing the four-week with the control group.

Finally, the most recent RCT [50] ($n = 63$) on the topic also evaluated the effect of different durations of testosterone pre-treatment on IVF outcomes of poor responders according to the Bologna criteria. Patients were randomized to eight weeks of pre-treatment with transdermal testosterone gel 12.5 mg/d, to 10 days of pre-treatment, and, finally, to no pre-treatment. The number of oocytes retrieved, and mature oocytes retrieved, did not differ between the groups compared.

Synthesis and critical appraisal of evidence

Multiple systematic reviews and meta-analyses on the use of pre-treatment testosterone have been published over the years [37, 38, 51]. The most recent published systematic review and meta-analysis analysed 8 RCTs, including 653 patients in total [52]. Based on the results of this meta-analysis, testosterone pre-treatment is associated with a higher number of oocytes retrieved (mean difference-MD: +0.94, 95% CI: 0.46–1.42). Clinical pregnancy rates (relative risk-RR: 2.07, 95% CI: 1.33–3.20) and live birth rates (RR: 2.09, 95% CI: 1.11–3.95) were also significantly higher in poor responders after testosterone pre-treatment compared to controls (Figure 55.2).

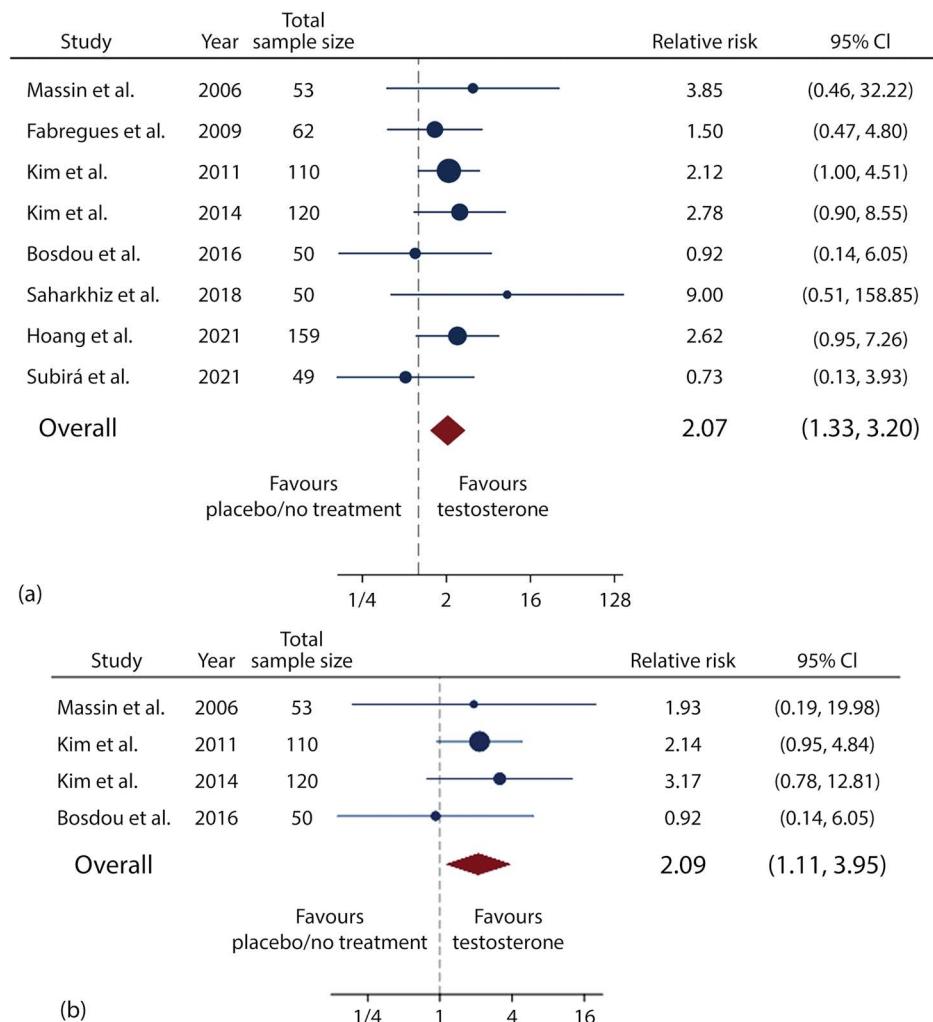


FIGURE 55.2 Statistical synthesis of studies evaluating the use of testosterone pre-treatment in poor responders based on Neves et al. (2022) [52]. (a) Clinical pregnancy. (b) Live birth.

Overall, the current literature on the use of testosterone pre-treatment suggests a beneficial effect, however the available studies are small with inherent limitations. There is a variation in the definition of poor responders, although, encouragingly, the last four RCTs have all utilized the Bologna criteria definition. Nevertheless, there is still substantial heterogeneity between these studies, both statistically and, most importantly, clinically, with differences in the daily dose of testosterone and the duration of the pre-treatment. The latter represents a topic of controversy, and further randomized evidence is required to settle this debate. Until such evidence becomes available, the use of pre-treatment testosterone for poor responders undergoing ovarian stimulation for ART cannot be confidently recommended. The 2020 ESHRE guidelines on ovarian stimulation for IVF/ICSI also state that

"Use of testosterone before ovarian stimulation is probably not recommended for poor responders" [53].

Dehydroepiandrosterone (DHEA)

Physiological basis of the intervention

DHEA is a steroid of the $\Delta 5$ pathway of steroidogenesis produced mainly in the zona reticularis of the adrenal cortex, but also in the gonads and the brain. It is a relatively weak form of androgen (with weak affinity to the androgen receptor), and for this reason it is considered mainly a precursor of more potent androgens, such as testosterone and DHT [54] (Figure 55.3). Along with its sulphated metabolite DHEA-S (with DHEA-S being by far the most abundant form), they are produced

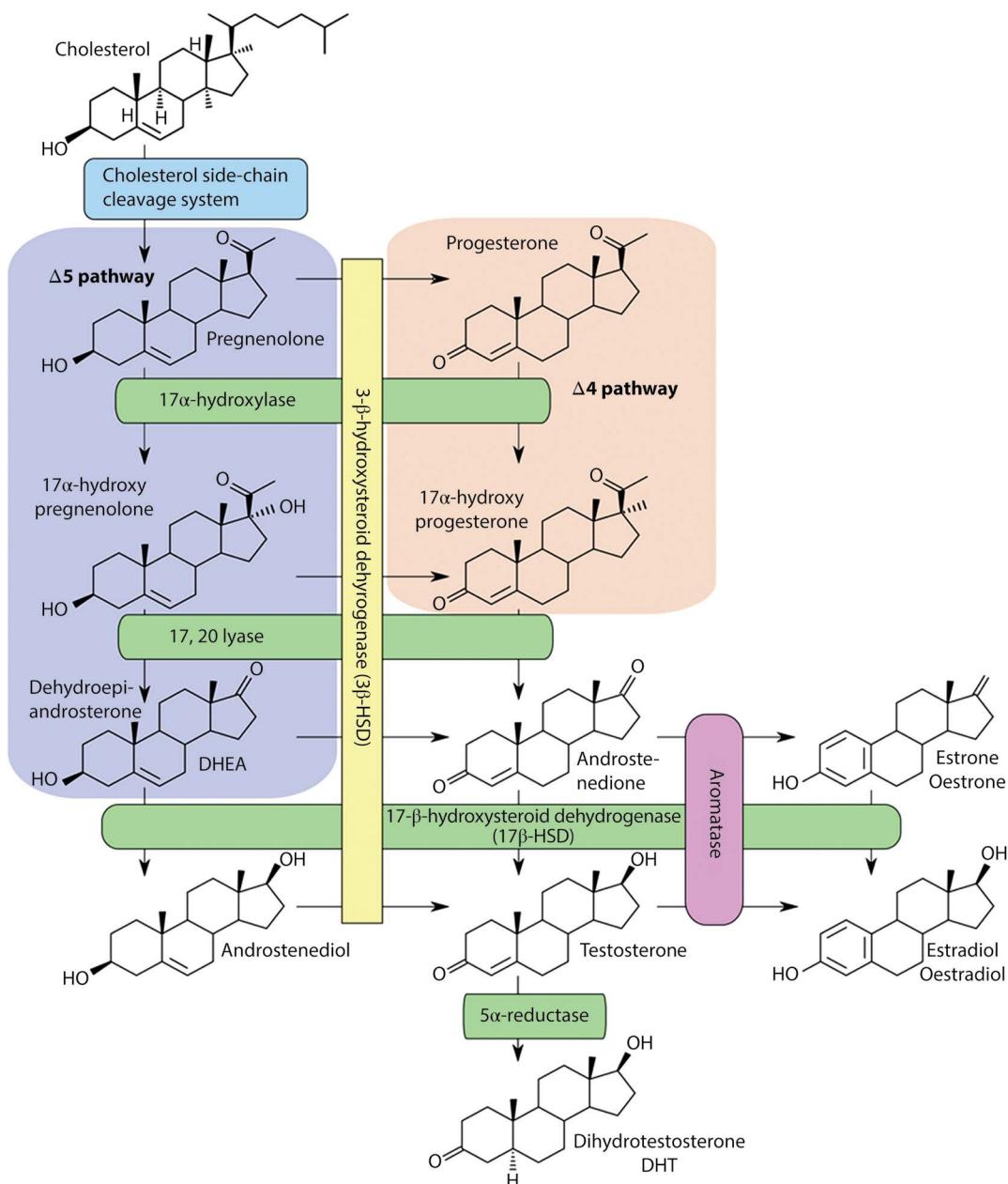


FIGURE 55.3 Steroidogenesis of androgens and oestrogen.

in large quantities throughout an individual's life with a peak around the third decade of life and a steady decline thereafter [55]. DHEA-S is much more strongly bound by albumin compared to DHEA and this affects their clearance rate, with DHEA having a half-life of one to three hours and DHEA-S of 10–20 hours [56]. A small portion of DHEA is also bound by SHBG (~5%–10%) and the remaining 3%–5% is free in the circulation. Exogenous administration of 50 mg DHEA/d in postmenopausal women has been shown to lead to an increase of serum DHEA and DHEA-S concentrations but also to a significant increase of serum testosterone (from 0.72 nmol/L to 1.46 nmol/L) and DHT (from 0.32 nmol/L to 0.9 nmol/L) [57]. Peak concentrations are usually achieved within two to four hours of administration [58].

There is experimental evidence to suggest that DHEA is produced in the theca cells of the ovary and can then act as a substrate for more potent androgen production and eventually conversion to oestrogens. However, there is also data supporting that DHEA-S from the circulation can also be used in the follicle as a substrate for production of more potent androgens [59]. Moreover, oral administration of DHEA has been shown to lead to a significant increase of serum IGF-I concentration [57, 60] which is known to increase FSH responsiveness of granulosa cells [61].

Clinical evidence

The first clinical evidence of a potential beneficial effect of DHEA administration in poor responders undergoing ovarian stimulation for IVF were provided by Casson et al. [40] in a case series of five women. These women were <41 years old with unexplained infertility who had previously exhibited poor response (fewer than three oocytes and peak oestradiol <500 pg/mL) and received 80 mg of oral micronized DHEA for two months. In the subsequent ovarian stimulation cycle, all five patients exhibited improved responsiveness to gonadotropins stimulation with peak oestradiol concentrations increasing by threefold compared to their original stimulation cycles.

This case series report sparked significant interest in the research community, and several studies were subsequently published, many of which were retrospective and employed a before-after design which has well-known methodological limitations.

In terms of RCTs, the first one to test the hypothesis was performed by Wiser et al. [62] and included women <42 years old with a poor response defined as retrieval of fewer than five oocytes, poor-quality embryos, or cycle cancellation due to poor response to ovarian stimulation when the starting dose of gonadotropins was ≥300 IU/d. It was a small RCT (n = 33) which randomized 17 patients to receive 75 mg/d of DHEA for at least six weeks before initiation of ovarian stimulation and 16 patients to the control group. Both groups were allowed to perform two stimulated cycles. Patients randomized in the DHEA group who did not conceive during their first cycle had subsequent DHEA pre-treatment for 16–18 weeks before their next stimulation cycle. The researchers supported that their data showed that patients in the DHEA group had significantly higher live birth rates compared to the control group (23.1% vs 4.0%; p = 0.05). However, this study's methodological design has been challenged, as there was no power analysis and the data included do not support the author's conclusions [63].

The study by Artini et al. [64] was an RCT in 24 poor responders according to the Bologna criteria. Patients in the DHEA group (n = 12) received 25 mg tds of DHEA for three months before ovarian stimulation. There were no significant differences in

the number of oocytes retrieved, the number of good quality embryos, and clinical pregnancy rates between the two groups.

On the other hand, the RCT by Moawad et al. [65] was performed on 133 patients with fewer than five oocytes retrieved or cycle cancellation due to poor response in a previous IVF cycle where ≥300 IU/d of FSH had been used. All patients were also required to have an AMH <1.7 µg/L. Patients were randomized to DHEA pre-treatment of 25 mg tds for at least 12 weeks (n = 67) or no pre-treatment (n = 66). The authors found that DHEA pre-treatment led to a significantly higher number of oocytes retrieved, lower cancellation rates, and a higher number of embryos transferred compared to the control group. There were no significant differences in terms of ongoing pregnancy rates.

The largest RCT (n = 208) evaluating the effect of DHEA pre-treatment in poor responders was published in 2014 by Kara et al. [66]. The population included in this study were women with serum AMH <1 ng/mL or serum FSH >15 IU/L and antral follicle count of less than four on day 2 of the menstrual cycle. Patients were randomized to receive 75 mg/d of DHEA for 12 weeks (n = 104), whereas women in the control group did not receive any pre-treatment (n = 104). There were no differences in the number of oocytes retrieved, fertilization rates, and the clinical pregnancy rates between the two groups. Therefore, this RCT challenged the notion that DHEA pre-treatment can be of value for poor responders.

Synthesis and critical appraisal of evidence

Several other RCTs have been subsequently published with mixed results, some in favour of DHEA use [67] and some not [68, 69]. Not surprisingly, multiple meta-analyses have been published on this topic with conflicting results [51, 70–73]. The most recent meta-analysis on the topic included eight RCTs (N = 727 patients, in total) and suggested there is no significant difference in the number of oocytes retrieved between poor responders who received DHEA pre-treatment and those who did not (mean difference: 0.76 oocytes; -0.35 to +1.88) [52]. Similarly, there were no differences in the clinical pregnancy rates (RR: 1.17, 95% CI: 0.87–1.57) and live birth rates (RR: 0.97, 95% CI: 0.47–2.01) between the two groups compared [52] (Figure 55.4).

Overall, the available literature on the value of DHEA pre-treatment in poor responders is characterized by limitations. Multiple retrospective studies have been published and these are particularly prone to various sources of bias. Importantly, the use of the before–after design is flawed for these types of comparisons as the “regression to the mean” effect leads to exaggerated effect sizes. Unfortunately, most of the available RCTs are small and of low methodological quality. There is considerable variability in the definition of poor response and further differences in the dose and duration of DHEA pre-treatment. Collectively, there is currently no good quality evidence to support the use of DHEA in poor responders. This might change with the accumulation of further evidence from future trials, which should also aim to elucidate the optimal dose and duration of DHEA pre-treatment. The 2020 ESHRE guidelines on ovarian stimulation for IVF/ICSI also state that “Use of dehydroepiandrosterone before and/or during ovarian stimulation is probably not recommended for poor responders” [53].

Conclusion

The topic of androgen pre-treatment in poor responders has attracted the attention of many researchers for more than 20 years. The two main androgens evaluated are transdermal

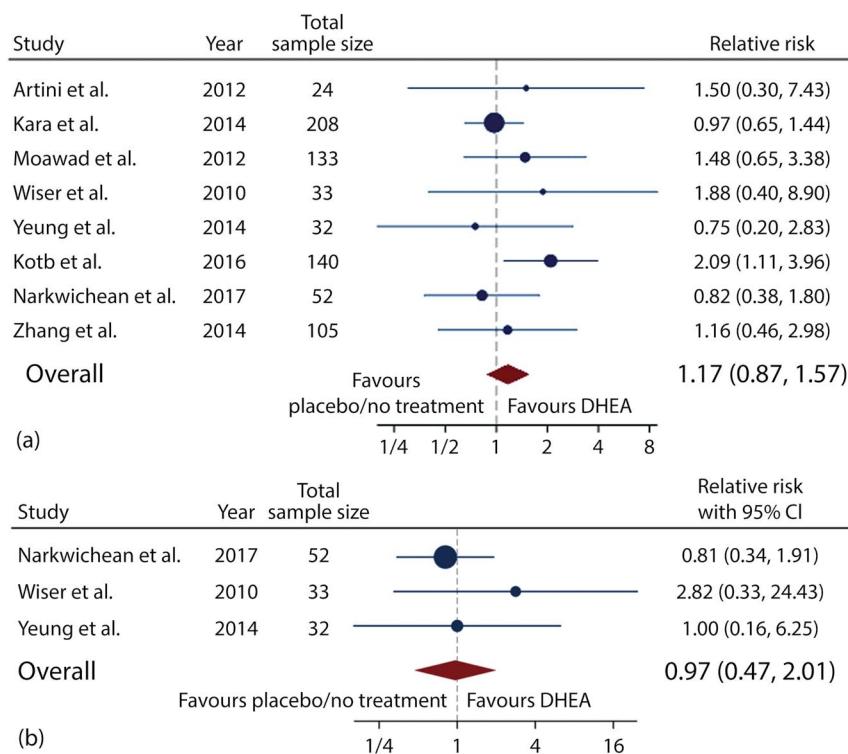


FIGURE 55.4 Statistical synthesis of studies evaluating the use of dehydroepiandrosterone (DHEA) pre-treatment in poor responders based on Neves et al. (2022) [52]. (a) Clinical pregnancy. (b) Live birth.

testosterone and oral DHEA. Unfortunately, the body of available evidence is not sufficient to confidently support or reject the benefit for either of these treatments. Future research should ensure the use of a well-defined population of poor responders within RCTs of high methodological quality, which evaluate both efficacy and safety of these interventions. Transdermal testosterone appears to be a more appealing candidate for future research as it has a more direct effect on the ovary and therefore is more likely to produce a clinical benefit, if such a benefit exists.

Growth hormone

Physiological basis of the intervention

Growth hormone (GH) is an anterior pituitary hormone (191 amino acids) that has several key roles in growth and development but also has been shown to be an important contributor to ovarian steroidogenesis and follicular development [74].

Animal studies on mice lacking GH receptor and GH-binding protein have shown that growth hormone is required for appropriate follicular development [75]. Moreover, GH increases the intraovarian production of IGF-I [76, 77], which is considered to play an important role in ovarian function, follicular recruitment, and protection from apoptosis, oestrogen production, and oocyte maturation [74, 78–80]. Therefore, it has been postulated that GH may positively affect folliculogenesis both qualitatively (oocytes of better quality) and quantitatively (increased number of oocytes), and this effect may be mediated by the increase of IGF-I [74].

Clinical evidence

The first report of the use of GH during ovarian stimulation was by Homburg et al. [81] in 1988. Four patients were administered

20 IU of human GH (hGH) on alternate days for two weeks while being stimulated with human menopausal gonadotropin (hMG). The authors observed that compared to previous cycles of the same patients, significantly less ampoules of hMG were required to complete ovarian stimulation.

These initial clinical data led to the first use of hGH in poor responders undergoing ovarian stimulation [82]. This was a case series in 10 previous poor responders who received 24 IU ($n = 5$) or 12 IU ($n = 5$) of hGH on alternate days starting on the same day as hMG administration. Comparing the outcome of the index stimulation cycle with their previous stimulated cycle, hGH administration was significantly associated with a shorter duration of stimulation, a lower total dose of hMG, and a higher number of oocytes retrieved.

The first RCT on this topic was published the same year by Owen et al. [83]. It randomized 25 patients, <38 years of age, with a prior poor response to ovarian stimulation defined as fewer than six oocytes retrieved with fewer than four embryos developed. Patients who were randomized to receive hGH ($n = 13$), were given 24 IU of hGH on alternate days during ovarian stimulation with hMG for a maximum of two weeks. Compared to the group who were randomized to receive placebo ($n = 12$), patients who received hGH appeared to require a lower number of hMG ampoules to complete ovarian stimulation and had significantly more 2PN oocytes after IVF. More oocytes were retrieved in the hGH group (median: 11) compared to the placebo group (median: 5.0) but this did not reach statistical significance.

On the other hand, the RCT by Bergh et al. [84] failed to show a beneficial effect. This was a double-blind, placebo-controlled RCT on 40 poor responders defined as women with fewer than five oocytes after adequate stimulation in two previous failed IVF

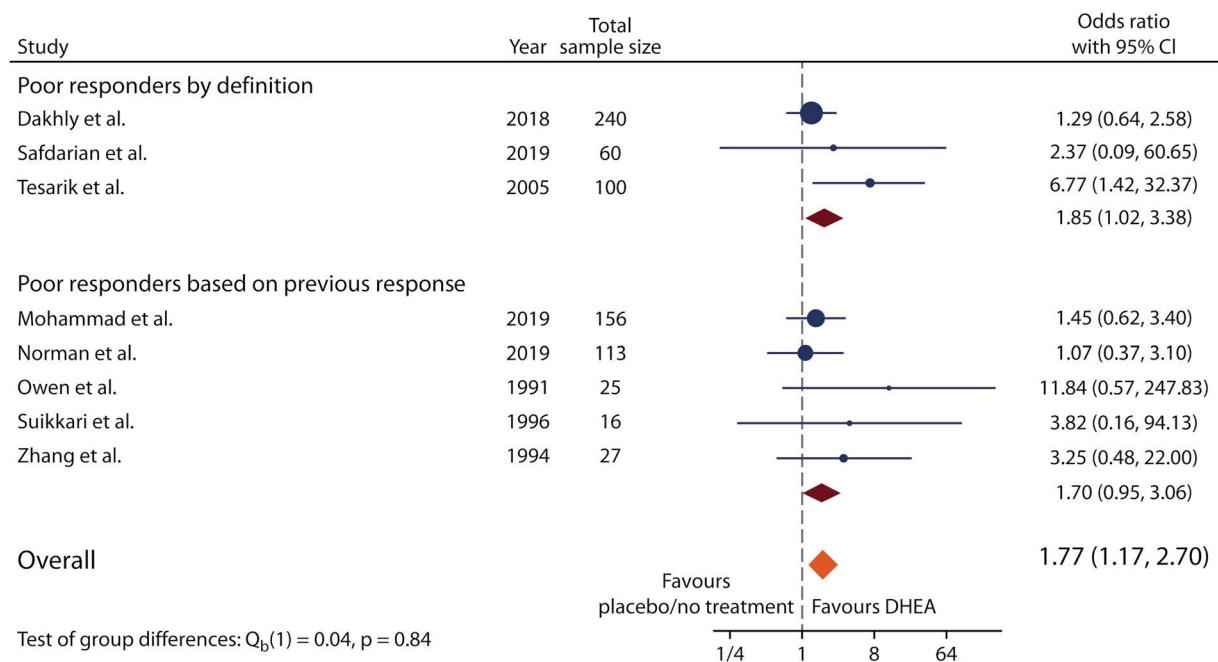


FIGURE 55.5 Statistical synthesis of studies evaluating the use of human growth hormone (hGH) pre-treatment in poor responders based on Sood et al. (2021) [90].

cycles. The researchers tested whether hGH co-administration with hMG is beneficial, but also whether hGH pre-treatment for seven days before hMG stimulation might be of value. Patients were randomized to receive placebo as pre-treatment and then hGH during ovarian stimulation as co-treatment ($n = 10$), placebo as pre-treatment and co-treatment ($n = 10$), hGH both as pre-treatment and during hMG stimulation ($n = 10$), and hGH as pre-treatment and placebo as co-treatment during hMG ($n = 10$). For those women who received hGH as co-treatment, the dose was 0.1 IU of hGH per kilogram of body weight per day, starting simultaneously with hMG until the day of triggering final oocyte maturation; the control group received placebo. The researchers were not able to detect any differences in terms of the total dose of hMG, the duration of stimulation, and the number of oocytes retrieved.

Synthesis and critical appraisal of evidence

A meta-analysis published in 2009 [85] analysed the six RCTs available at the time and was the first to clearly suggest a potential beneficial effect of hGH co-treatment in poor responders undergoing ovarian stimulation for IVF. Several new RCTs followed, some of which supported a beneficial effect of hGH co-administration during ovarian stimulation of poor responders [86–88], while others did not [89].

A Cochrane systematic review and meta-analysis published in 2021 identified 14 RCTs performed in poor responders ($n = 1282$), testing the value of hGH addition during ovarian stimulation [90]. The poor responders in the individual RCTs were defined according to various criteria which could be grouped in expected poor ovarian response based on age or other tests suggesting low ovarian response, in proven poor responders based on previous low response to ovarian stimulation, and in those studies which use the Bologna criteria, which encompass both expected and proven poor responders. The dose of hGH was also highly variable, ranging from 4 IU per day [91] to 24 IU every alternate day [83].

Based on the results of the meta-analysis, there was a significant increase in the probability of live birth rate in poor responders who received hGH compared to those who did not (OR: 1.77, 95% CI: 1.17–2.70; $n = 8$ RCTs, 837 patients) (Figure 55.5). There was no significant difference in the effect size between studies using proven or expected poor responders (by definition). Compared to the control group, women who received hGH had a significant decrease in the mean total dose of gonadotropins required to complete ovarian stimulation (MD: -1088 IU, 95% CI: -1203 to -973) and an increase in the number of oocytes retrieved (mean difference: +1.40 oocytes, 95% CI: 1.16–1.64). Despite these encouraging results, the authors of the review note the relatively small size of the total body of evidence, the heterogeneity in the populations included, and the clinical protocols used in the eligible studies. These limitations render the results of this meta-analysis of very low certainty and therefore these should be interpreted with caution [90].

Conclusion

Growth hormone co-administration during ovarian stimulation was proposed more than 30 years ago as a potentially useful adjunct. Nevertheless, the RCTs that have been performed are scarce and have methodological pitfalls. The optimal clinical protocol for hGH administration is also not universally agreed on and this creates further issues in the interpretation of the available evidence. Although the evidence is intriguing and should form the base for further research, it is still insufficient to recommend the use of hGH in poor responders. The side effect profile of hGH administration along with its high cost are additional factors that should be considered and deter the use of hGH for the treatment of poor responders outside the context of well-designed clinical trials. For these reasons, the 2020 ESHRE guidelines on ovarian stimulation for IVF/ICSI also state that “Use of adjuvant GH before and/or during ovarian stimulation is probably not recommended for poor responders.” [53].

Antioxidants

Physiological basis of the intervention

As discussed previously, one of the strategies of improving the outcomes for poor responders undergoing ovarian stimulation for IVF has been attempting to improve the quality of the oocytes, therefore potentially increasing the probability of obtaining good quality embryos and achieving a pregnancy. Although a diminished ovarian reserve does not necessarily translate to oocytes of inferior quality, this can be true when there are relevant underlying factors such as advanced female age [92], prior gonadotoxic therapy [93], or endometriosis [94]. Importantly, though, in the presence of a small number of oocytes, the prognostic effect of oocyte quality is magnified.

Oxidative stress is caused by increased concentrations of reactive oxygen species (ROS), which are a by-product of the aerobic cellular metabolism [95]. ROS have been shown to cause DNA damage, lipid peroxidation, and protein damage. They are also produced in the ovary and seem to have a role in follicular atresia and ovulation [96]. Notably, oxidative stress has been shown to inhibit oocyte development [97], cause meiotic arrest [98], and induce oocyte apoptosis [97].

For these reasons, oxidative stress has been suggested to be an important factor of oocyte quality, particularly in the context of ovarian stimulation, where it has been shown that ovarian stimulation protocols can lead to significant changes in the antioxidant capacity of the follicle and render the oocyte susceptible to oxidative damage [99, 100].

Antioxidants are biological or chemical substances that inhibit oxidation and are ROS scavengers. It is a diverse group of organic nutrients that includes vitamins, minerals, and polyunsaturated fatty acids. They are produced endogenously but can also be administered exogenously. The administration of several different antioxidants, either alone or in combination, has, therefore, been proposed as a potential strategy to increase oocyte quality and enhance the outcome of IVF both in unselected patients and in patients with previous poor outcomes, including poor ovarian response [101]. These include L-arginine, vitamin E, myo-inositol, D-chiro-inositol, carnitine, selenium, vitamin B complex, vitamin C, vitamin D and calcium, CoQ10, melatonin, folic acid, and omega-3 polyunsaturated fatty acids [101].

Based on a recent Cochrane systematic review and meta-analysis, CoQ10, melatonin, and L-arginine have been tested with RCTs in poor responders [101]. In the following section the clinical evidence on the use of these antioxidants in poor responders will be reviewed.

CoQ10

Physiological basis of the intervention

Coenzyme Q10, also known as ubiquinol, is considered a free radical scavenging antioxidant and has a central role to the electron transport chain. Experimental evidence suggests that female aging can lead to mitochondrial dysfunction of the granulosa cells due to a CoQ10 deficit [102]. *In vitro* studies in mice have shown that supplementation of CoQ10 can restore age-induced deterioration of oocyte quality by protecting them from high level oxidative stress and DNA damage to avoid apoptosis [103].

CoQ10 is also produced as a dietary supplement, and it has been shown to reach peak concentrations at two to six hours after oral administration. It is currently not approved by the FDA for the treatment of any medical condition.

Clinical evidence

The use of CoQ10 supplementation in the context of ART was first proposed in an RCT performed on women 35–43 years old undergoing ovarian stimulation [104]. Patients were randomized to either two months of 600 mg/d CoQ10 pre-treatment (n = 22) or placebo (n = 19). The study aimed at assessing the post-meiotic aneuploidy rate of the oocytes but had to be prematurely terminated due to safety concerns regarding polar body biopsy. Eventually, only 27 patients completed the study protocol, and no significant differences were shown in the post-meiotic aneuploidy rate between women who received CoQ10 and those who received placebo.

The first RCT to evaluate the effect of CoQ10 administration in poor responders during ovarian stimulation for ART was announced and published only as an abstract in the meeting proceedings of the Annual Conference of the American Society for Reproductive Medicine in 2016 [105]. This RCT included 78 poor responders according to the Bologna criteria aged 36–40 years. Patients were randomized to receive 600 mg of CoQ10 twice a day (1200 mg daily dose) for 12 weeks (n = 39) or no pre-treatment (n = 39). There were no significant differences in the number of MII oocytes retrieved (1.82 vs 1.87; p = 0.77), implantation rate, and clinical pregnancy rate (fetal heartbeat at seven weeks) (15.4% vs 12.8%; p = 0.64) between the two groups compared.

The next RCT performed on this clinical question was published as a full text by Xu et al. in 2018 [104]. This study included young poor responders (n = 186) age <35 years, AMH <1.2 ng/mL, and antral follicle count (AFC) <5, consistent with group 3 of the POSEIDON stratification. The study group received 200 mg tds of CoQ10 (600 mg/d) for 60 days before commencing ovarian stimulation, while the control group did not receive placebo. The primary outcome measure of the study was the number of high-quality day-3 embryos. Women who received pre-treatment with CoQ10 required a lower total dose of gonadotropins to complete ovarian stimulation and had a higher number of oocytes retrieved compared to the control group (median: 4 vs 2, respectively; p = 0.002). The number of high-quality day-3 embryos was also significantly higher in the study group (median: 1 vs 0; p = 0.03). Significantly more patients had an oocyte retrieval without reaching an embryo transfer in the control group and the study group had a significantly higher number of patients with cryopreserved embryos. There were no significant differences between the two groups compared in terms of pregnancy outcomes either per first embryo transfer or cumulative per stimulated cycle [106].

Overall, the clinical evidence around the potential benefit of CoQ10 pre-treatment is scarce and conflicting; therefore, its use in clinical practice cannot be recommended.

Melatonin

Physiological basis of the intervention

Melatonin is a hormone mainly synthesized and secreted by the pineal gland and has a diurnal variation with peak concentrations observed between midnight and early morning [107]. One of its main functions is to regulate circadian rhythm and the sleep-wake cycle [108]. However, melatonin has been recently suggested to have important antioxidative properties, with some considering it superior to traditional antioxidants. Melatonin exerts antioxidant effects through its receptors MT1 and MT2 but also can act as a direct free radical scavenger [109].

Melatonin receptors have been found in granulosa cells [110, 111], and melatonin has been shown to have direct effects on ovarian function [111]. Melatonin concentrations are high in the

follicular fluid and seem to be higher in large follicles, and it has been postulated that melatonin, through its antioxidative function, can rescue developing follicles from atresia [112].

A series of animal studies have demonstrated the significance of the role of melatonin in reproductive function and the positive effect it can exert on oocyte maturation and embryo quality [109].

Based on this evidence, researchers have hypothesized that administration of melatonin can also positively affect human reproduction and the outcome of ART.

Clinical evidence

General ART population

In 2008, Tamura et al. [113] supported that administration of 3 mg/d of melatonin in women undergoing ART from the fifth day of the previous menstrual cycle until the day of oocyte retrieval led to a significant increase in the intrafollicular concentration of melatonin, reduced the intrafollicular concentration of markers of oxidative stress, and improved fertilization rates.

Eryilmaz et al. [114] performed an RCT of melatonin administration in ART patients with sleeping problems using the protocol described in the Tamura et al. [113] study and although they did not observe any improvement in the sleep quality, they did observe a significantly higher number of retrieved oocytes, MII oocytes, and a good-quality embryo rate in women who received melatonin. In terms of evidence from RCTs in general ART population, Batioglu et al. [115] randomized 85 women to either 3 mg/d of melatonin ($n = 40$) or no co-treatment ($n = 45$). There were no significant differences between the two groups in terms of the number of oocytes retrieved, fertilization rates, and clinical pregnancy rates, although the authors did observe a significantly higher percentage of MII oocytes (81.9% vs 75.8%; $p = 0.034$) and a higher number of good-quality embryos (median: 3.2 vs 2.5; $p = 0.035$) in the melatonin group.

Finally, Fernando et al. performed a four-arm RCT of women having their first ART cycle and compared the value of administering three different doses of melatonin from day 2 of stimulation until the day of oocyte retrieval, i.e. 2 mg/d ($n = 41$), 4 mg/d ($n = 39$), 8 mg/d ($n = 40$), with placebo ($n = 40$) [116]. The authors did not observe significant differences between the groups in total oocyte number, number of MII oocytes, number of fertilized oocytes, or the number or quality of embryos. Moreover, there were no differences in clinical pregnancy or live birth rates.

Poor responders

Only one RCT has tested this intervention in 80 women with diminished ovarian reserve, defined as the presence of two of the following three criteria: (i) AMH ≤ 1 pmol/L, (ii) FSH ≥ 10 IU/L, and (iii) bilateral antral follicle count ≤ 6 [117]. Participants were randomized to receive either melatonin 3 mg/d from the fifth day of the previous menstrual cycle until the day of oocyte retrieval ($n = 40$) or placebo ($n = 40$). All patients underwent ovarian stimulation using recombinant gonadotropins and a midluteal long downregulation GnRH agonist protocol. There were no significant differences in terms of duration of ovarian stimulation or total dose of gonadotropins required. The serum oestradiol on the day of hCG administration was significantly higher in the melatonin group. The number of MII oocytes was higher in the melatonin group compared to the placebo group (mean: 5.4 vs 3.7 oocytes, respectively; $p = 0.054$), but this difference did not reach statistical significance. No significant differences were observed in clinical pregnancy rates between the groups compared.

Therefore, despite some promising early clinical data (particularly as it pertains to number of MII oocytes), the only randomized study evaluating the use of melatonin in women with diminished ovarian reserve has not been able to demonstrate a clear clinical benefit. Therefore, further evidence is required for solid conclusions to be drawn and until such evidence is available, it remains uncertain whether a true benefit exists.

L-arginine

Physiological basis of the intervention

L-arginine is an α -amino acid which is involved in several metabolic processes in the human body. One of the most intriguing roles of L-arginine is its close relationship with the important signal molecule nitric oxide (NO), being the only substrate in its biosynthesis. Nitric oxide has been shown to have a role in various metabolic processes taking place in the human ovary, including folliculogenesis and oocyte meiotic maturation [118]. There is also evidence to suggest that NO has an important role in periovulatory vasodilatory modulation of rat ovarian blood flow [119].

Clinical evidence

On the basis of these roles of NO, L-arginine has been proposed as a potential adjvant for the treatment of poor responders. Only one RCT published in 1999 has evaluated the role of this intervention in the treatment of poor responders [120]. This study randomized 34 poor responders who had previously had a cancelled cycle due to serum oestradiol concentration <1100 pmol/L and/or fewer than three follicles on day 8 of ovarian stimulation. Patients in the study group ($n = 17$) received 16 gr of oral L-arginine per day in addition to their gonadotropins during ovarian stimulation while women in the control group received only gonadotropins. No significant differences were observed in the total dose of gonadotropins or the duration of ovarian stimulation between the two groups. The L-arginine group had significantly more oocytes retrieved (4.1 vs 1.6; $p = 0.049$). The number of transferred embryos was also higher in the L-arginine group, but this was not statistically significant.

No further RCTs have examined the potential benefit of this intervention in poor responders and therefore there is currently insufficient evidence to support its use in clinical practice.

Conclusion

The administration of antioxidants during the treatment of poor responders or women with diminished ovarian reserve for the improvement ART outcome has been proposed based on data suggesting the importance of antioxidant activity in the process of folliculogenesis and oocyte maturation as well as initial evidence data from animal studies. Nevertheless, high quality evidence from clinical studies in humans is not available and therefore their use outside the context of well-designed clinical studies should be discouraged.

Summary

Poor ovarian response represents a significant therapeutic conundrum in ART, which has been challenging clinicians and researchers for decades. Several interventions, called "adjutants" or "add-ons," have been proposed to improve the outcome of ART in these patients. These include androgens such as testosterone or DHEA, growth hormone, as well as different antioxidants. Whether such interventions are truly beneficial for this

population can only be properly assessed with an RCT. A review of the available randomized evidence suggests that none of these interventions has the evidence base to recommend their adoption in clinical practice.

Future studies should focus on testing add-ons with strong physiological plausibility in a sufficiently homogeneous population of poor responders while using properly developed clinical protocols in terms of dosage and duration. The main outcome of interest in this research should always be live birth rates after ART, while also prioritizing the evaluation of the safety of these interventions.

References

- Sunkara SK, LaMarca A, Polyzos NP, Seed PT, Khalaf Y. Live birth and perinatal outcomes following stimulated and unstimulated IVF: Analysis of over two decades of a nationwide data. *Hum Reprod*. 2016;31(10):2261–7.
- Law YJ, Zhang N, Kolibianakis EM, Costello MF, Keller E, Chambers GM, et al. Is there an optimal number of oocytes retrieved at which live birth rates or cumulative live birth rates per aspiration are maximized after ART? A systematic review. *Reprod Biomed Online*. 2021;42(1):83–104.
- Tarlatzis TB, Venetis CA, Devreker F, Englert Y, Delbaere A. What is the best predictor of severe ovarian hyperstimulation syndrome in IVF? A cohort study. *J Assist Reprod Genet*. 2017;34(10):1341–51.
- Garcia JE, Jones GS, Acosta AA, Wright G Jr. Human menopausal gonadotropin/human chorionic gonadotropin follicular maturation for oocyte aspiration: Phase II, 1981. *Fertil Steril*. 1983;39(2):174–9.
- Tarlatzis BC, Zepiridis L, Grimbizis G, Bontis J. Clinical management of low ovarian response to stimulation for IVF: A systematic review. *Hum Reprod Update*. 2003;9(1):61–76.
- Keay SD, Liversedge NH, Mathur RS, Jenkins JM. Assisted conception following poor ovarian response to gonadotrophin stimulation. *Br J Obstet Gynaecol*. 1997;104(5):521–7.
- Akande VA, Fleming CF, Hunt LP, Keay SD, Jenkins JM. Biological versus chronological ageing of oocytes, distinguishable by raised FSH levels in relation to the success of IVF treatment. *Hum Reprod*. 2002;17(8):2003–8.
- Nargund G, Bromhan D. Comparison of endocrinological and clinical profiles and outcome of IVF cycles in patients with one ovary and two ovaries. *J Assist Reprod Genet*. 1995;12(7):458–60.
- Keay SD, Liversedge NH, Jenkins JM. Could ovarian infection impair ovarian response to gonadotrophin stimulation? *Br J Obstet Gynaecol*. 1998;105(3):252–3.
- Das M, Shehata F, Son WY, Tulandi T, Holzer H. Ovarian reserve and response to IVF and in vitro maturation treatment following chemotherapy. *Hum Reprod*. 2012;27(8):2509–14.
- Loh S, Wang JX, Matthews CD. The influence of body mass index, basal FSH and age on the response to gonadotrophin stimulation in non-polycystic ovarian syndrome patients. *Hum Reprod*. 2002;17(5):1207–11.
- Bibi G, Malcov M, Yuval Y, Reches A, Ben-Yosef D, Almog B, et al. The effect of CGG repeat number on ovarian response among fragile X premutation carriers undergoing preimplantation genetic diagnosis. *Fertil Steril*. 2010;94(3):869–74.
- Ruth KS, Day FR, Hussain J, Martinez-Marchal A, Aiken CE, Azad A, et al. Genetic insights into biological mechanisms governing human ovarian ageing. *Nature*. 2021;596(7872):393–7.
- Venetis CA, Kolibianakis EM, Tarlatzis TB, Tarlatzis BC. Evidence-based management of poor ovarian response. *Ann N Y Acad Sci*. 2010;1205:199–206.
- Kyrou D, Kolibianakis EM, Venetis CA, Papanikolaou EG, Bontis J, Tarlatzis BC. How to improve the probability of pregnancy in poor responders undergoing in vitro fertilization: A systematic review and meta-analysis. *Fertil Steril*. 2009;91(3):749–66.
- Polyzos NP, Devroey P. A systematic review of randomized trials for the treatment of poor ovarian responders: Is there any light at the end of the tunnel? *Fertil Steril*. 2011;96(5):1058–61 e7.
- Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L, et al. ESHRE consensus on The definition of 'poor response' to ovarian stimulation for in vitro fertilization: The Bologna criteria. *Hum Reprod*. 2011;26(7):1616–24.
- Papathanasiou A. Implementing the ESHRE 'poor responder' criteria in research studies: Methodological implications. *Hum Reprod*. 2014;29(9):1835–8.
- Venetis CA. The Bologna criteria for poor ovarian response: The good, The bad and The way forward. *Hum Reprod*. 2014;29(9):1839–41.
- Ferraretti AP, Gianaroli L. The Bologna criteria for the definition of poor ovarian responders: Is there a need for revision? *Hum Reprod*. 2014;29(9):1842–5.
- Humaidan P, Alviggi C, Fischer R, Esteves SC. The novel POSEIDON stratification of 'Low prognosis patients in assisted reproductive Technology' and its proposed marker of successful outcome. *F1000Res*. 2016;5:2911.
- Human Fertilisation & Embryology Authority. Treatment add-ons with limited evidence 2022. Available from: <https://www.hfea.gov.uk/treatments/treatment-add-ons/>.
- Lensen S, Hammarberg K, Polyakov A, Wilkinson J, Whyte S, Peate M, et al. How common is add-on use and how do patients decide whether to use them? A national survey of IVF patients. *Hum Reprod*. 2021;36(7):1854–61.
- Liperis G, Fraire-Zamora JJ, Makieva S, Massarotti C, Ali ZE, Kohlhepp F, et al. #ESHREjc report: Trick or treatment-evidence based use of add-ons in ART and patient perspectives. *Hum Reprod*. 2022;37(2):386–8.
- Weil SJ, Vendola K, Zhou J, Adesanya OO, Wang J, Okafor J, et al. Androgen receptor gene expression in the primate ovary: Cellular localization, regulation, and functional correlations. *J Clin Endocrinol Metab*. 1998;83(7):2479–85.
- Vendola K, Zhou J, Wang J, Famuyiwa OA, Bievre M, Bondy CA. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. *Biol Reprod*. 1999;61(2):353–7.
- Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA. Androgens stimulate early stages of follicular growth in the primate ovary. *J Clin Invest*. 1998;101(12):2622–9.
- Weil S, Vendola K, Zhou J, Bondy CA. Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. *J Clin Endocrinol Metab*. 1999;84(8):2951–6.
- Hillier SG, Tetsuka M, Fraser HM. Location and developmental regulation of androgen receptor in primate ovary. *Hum Reprod*. 1997;12(1):107–11.
- Nielsen ME, Rasmussen IA, Kristensen SG, Christensen ST, Mollgard K, Wreford Andersen E, et al. In human granulosa cells from small antral follicles, androgen receptor mRNA and androgen levels in follicular fluid correlate with FSH receptor mRNA. *Mol Hum Reprod*. 2011;17(1):63–70.
- Hillier SG, De Zwart FA. Evidence that granulosa cell aromatase induction/activation by follicle-stimulating hormone is an androgen receptor-regulated process in-vitro. *Endocrinology*. 1981;109(4):1303–5.
- Harlow CR, Hillier SG, Hodges JK. Androgen modulation of follicle-stimulating hormone-induced granulosa cell steroidogenesis in the primate ovary. *Endocrinology*. 1986;119(3):1403–5.
- Futterweit W, Deligdisch L. Histopathological effects of exogenously administered testosterone in 19 female to male transsexuals. *J Clin Endocrinol Metab*. 1986;62(1):16–21.
- Spinder T, Spijkstra JJ, van den Tweel JG, Burger CW, van Kessel H, Hompes PG, et al. The effects of long term testosterone administration on pulsatile luteinizing hormone secretion and on ovarian histology in eugonadal female to male transsexual subjects. *J Clin Endocrinol Metab*. 1989;69(1):151–7.

35. Hugues JN, Durnerin IC. Impact of androgens on fertility - physiological, clinical and therapeutic aspects. *Reprod Biomed Online.* 2005;11(5):570–80.
36. Frattarelli JL, Peterson EH. Effect of androgen levels on in vitro fertilization cycles. *Fertil Steril.* 2004;81(6):1713–4.
37. Bosdou JK, Venetis CA, Kolibianakis EM, Toulis KA, Goulis DG, Zepiridis L, et al. The use of androgens or androgen-modulating agents in poor responders undergoing in vitro fertilization: A systematic review and meta-analysis. *Hum Reprod Update.* 2012;18(2):127–45.
38. Sunkara SK, Pundir J, Khalaf Y. Effect of androgen supplementation or modulation on ovarian stimulation outcome in poor responders: A meta-analysis. *Reprod Biomed Online.* 2011;22(6):545–55.
39. Balasch J, Fábregues F, Peñarrubia J, Carmona F, Casamitjana R, Creus M, et al. Pretreatment with transdermal testosterone may improve ovarian response to gonadotrophins in poor-responder IVF patients with normal basal concentrations of FSH. *Hum Reprod.* 2006;21(7):1884–93.
40. Casson PR, Lindsay MS, Pisarska MD, Carson SA, Buster JE. Dehydroepiandrosterone supplementation augments ovarian stimulation in poor responders: A case series. *Hum Reprod.* 2000;15(10):2129–32.
41. Astapova O, Minor BMN, Hammes SR. Physiological and pathological androgen actions in the ovary. *Endocrinology.* 2019;160(5):1166–74.
42. Singh AB, Lee ML, Sinha-Hikim I, Kushnir M, Meikle W, Rockwood A, et al. Pharmacokinetics of a testosterone gel in healthy postmenopausal women. *J Clin Endocrinol Metab.* 2006;91(1):136–44.
43. Slater CC, Souter I, Zhang C, Guan C, Stanczyk FZ, Mishell DR. Pharmacokinetics of testosterone after percutaneous gel or buccal administration. *Fertil Steril.* 2001;76(1):32–7.
44. Massin N, Cedrin-Durnerin I, Coussieu C, Galey-Fontaine J, Wolf JP, Hugues JN. Effects of transdermal testosterone application on the ovarian response to FSH in poor responders undergoing assisted reproduction technique—a prospective, randomized, double-blind study. *Hum Reprod.* 2006;21(5):1204–11.
45. Kim CH, Howles CM, Lee HA. The effect of transdermal testosterone gel pretreatment on controlled ovarian stimulation and IVF outcome in low responders. *Fertil Steril.* 2011;95(2):679–83.
46. Kim CH, Ahn JW, Moon JW, Kim SH, Chae HD, Kang BM. Ovarian features after 2 weeks, 3 weeks and 4 weeks transdermal testosterone gel treatment and their associated effect on IVF outcomes in poor responders. *Dev Reprod.* 2014;18(3):145–52.
47. Bosdou JK, Venetis CA, Dafopoulos K, Zepiridis L, Chatzimeletiou K, Anifandis G, et al. Transdermal testosterone pretreatment in poor responders undergoing ICSI: A randomized clinical trial. *Hum Reprod.* 2016;31(5):977–85.
48. Saharkhiz N, Zademodares S, Salehpour S, Hosseini S, Nazari L, Tehrani HG. The effect of testosterone gel on fertility outcomes in women with a poor response in in vitro fertilization cycles: A pilot randomized clinical trial. *J Res Med Sci.* 2018;23:3.
49. Hoang QH, Ho HS, Do HT, Nguyen TV, Nguyen HP, Le MT. Therapeutic effect of prolonged testosterone pretreatment in women with poor ovarian response: A randomized control trial. *Reprod Med Biol.* 2021 Mar;27(20):305–12.
50. Subirà J, Algabe A, Vázquez S, Taroncher Dasí R, Mollá Robles G, Monzó Fabuel S, Baydal V, Ruiz Herreros A, García Camuñas N, Rubio Rubio JM. Testosterone does not improve ovarian response in Bologna poor responders: A randomized controlled trial (TESTOPRIM). *Reprod Biomed Online.* 2021 Sep;43(3):466–74.
51. Nagels HE, Rishworth JR, Siristatidis CS, Kroon B. Androgens (dehydroepiandrosterone or testosterone) for women undergoing assisted reproduction. *Cochrane Database Syst Rev.* 2015(11):CD009749.
52. Neves AR, Montoya-Botero P, Polyzos NP. Androgens and diminished ovarian reserve: The long road from basic science to clinical implementation. A comprehensive and systematic review with meta-analysis. *Am J Obstet Gynecol.* 2022;227(3):401–13.e18.
53. Ovarian Stimulation T, Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI(†). *Hum Reprod Open.* 2020;2020(2):hoaa009.
54. Kroboth PD, Salek FS, Pittenger AL, Fabian TJ, Frye RF. DHEA and DHEA-s: A review. *J Clin Pharmacol.* 1999;39(4):327–48.
55. Labrie F, Belanger A, Cusan L, Gomez JL, Candas B. Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. *J Clin Endocrinol Metab.* 1997;82(8):2396–402.
56. Rosenfeld RS, Rosenberg BJ, Fukushima DK, Hellman L. 24-hour secretory pattern of dehydroisoandrosterone and dehydroisoandrosterone sulfate. *J Clin Endocrinol Metab.* 1975;40(5):850–5.
57. Morales AJ, Nolan JJ, Nelson JC, Yen SS. Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. *J Clin Endocrinol Metab.* 1994;78(6):1360–7.
58. Arlt W, Justl HG, Callies F, Reincke M, Hubler D, Oettel M, et al. Oral Dehydroepiandrosterone for adrenal androgen replacement: Pharmacokinetics and peripheral conversion to androgens and estrogens in young healthy females after dexamethasone suppression. *J Clin Endocrinol Metab.* 1998;83(6):1928–34.
59. Haning RV Jr., Flood CA, Hackett RJ, Loughlin JS, McClure N, Longcope C. Metabolic clearance rate of dehydroepiandrosterone sulfate, its metabolism to testosterone, and its intrafollicular metabolism to dehydroepiandrosterone, androstenedione, testosterone, and dihydrotestosterone in vivo. *J Clin Endocrinol Metab.* 1991;72(5):1088–95.
60. Diamond P, Cusan L, Gomez JL, Belanger A, Labrie F. Metabolic effects of 12-month percutaneous dehydroepiandrosterone replacement therapy in postmenopausal women. *J Endocrinol.* 1996;150(Suppl):S43–50.
61. Monget P, Bondy C. Importance of the IGF system in early folliculogenesis. *Mol Cell Endocrinol.* 2000;163(1–2):89–93.
62. Wiser A, Gonen O, Gheler Y, Shavit T, Berkovitz A, Shulman A. Addition of dehydroepiandrosterone (DHEA) for poor-responder patients before and during IVF treatment improves the pregnancy rate: A randomized prospective study. *Hum Reprod.* 2010;25(10):2496–500.
63. Kolibianakis EM, Venetis CA, Tarlatzis BC. DHEA administration in poor responders. *Hum Reprod.* 2011;26(3):730–1.
64. Artini PG, Simi G, Ruggiero M, Pinelli S, Di Berardino OM, Papini F, et al. DHEA supplementation improves follicular micro-environment in poor responder patients. *Gynecol Endocrinol.* 2012;28(9):669–73.
65. Moawad A, Shaeer M. Long-term androgen priming by use of dehydroepiandrosterone (DHEA) improves IVF outcome in poor-responder patients. A randomized controlled study. *Mid East Fertil Soc J.* 2012;17(4):268–74.
66. Kara M, Aydin T, Aran T, Turktekin N, Ozdemir B. Does dehydroepiandrosterone supplementation really affect IVF-ICSI outcome in women with poor ovarian reserve? *Eur J Obstet Gynecol Reprod Biol.* 2014;173:63–5.
67. Kotb MM, Hassan AM, AwadAllah AM. Does dehydroepiandrosterone improve pregnancy rate in women undergoing IVF/ICSI with expected poor ovarian response according to the Bologna criteria? A randomized controlled trial. *Eur J Obstet Gynecol Reprod Biol.* 2016;200:11–5.
68. Narkwichean A, Maalouf W, Baumgarten M, Polanski L, Rainey-Fenning N, Campbell B, et al. Efficacy of dehydroepiandrosterone (DHEA) to overcome the effect of ovarian ageing (DITTO): A proof of principle double blinded randomized placebo controlled trial. *Eur J Obstet Gynecol Reprod Biol.* 2017;218:39–48.

69. Yeung TW, Chai J, Li RH, Lee VC, Ho PC, Ng EH. A randomized, controlled, pilot trial on the effect of dehydroepiandrosterone on ovarian response markers, ovarian response, and in vitro fertilization outcomes in poor responders. *Fertil Steril.* 2014;102(1):108–15.e1.
70. Li J, Yuan H, Chen Y, Wu H, Wu H, Li L. A meta-analysis of dehydroepiandrosterone supplementation among women with diminished ovarian reserve undergoing in vitro fertilization or intracytoplasmic sperm injection. *Int J Gynaecol Obstet.* 2015;131(3):240–5.
71. Narkwichean A, Maalouf W, Campbell BK, Jayaprakasan K. Efficacy of dehydroepiandrosterone to improve ovarian response in women with diminished ovarian reserve: A meta-analysis. *Reprod Biol Endocrinol.* 2013;11:44.
72. Schwarze JE, Canales J, Crosby J, Ortega-Hrepich C, Villa S, Pommer R. DHEA use to improve likelihood of IVF/ICSI success in patients with diminished ovarian reserve: A systematic review and meta-analysis. *JBRA Assist Reprod.* 2018;22(4):369–74.
73. Xu L, Hu C, Liu Q, Li Y. The effect of dehydroepiandrosterone (DHEA) supplementation on IVF or ICSI: A meta-analysis of randomized controlled trials. *Geburtshilfe Frauenheilkd.* 2019;79(7):705–12.
74. Hull KL, Harvey S. Growth hormone: Roles in female reproduction. *J Endocrinol.* 2001;168(1):1–23.
75. Bachelot A, Monget P, Imbert-Bonnre P, Coshigano K, Kopchick JJ, Kelly PA, et al. Growth hormone is required for ovarian follicular growth. *Endocrinology.* 2002;143(10):4104–12.
76. Hsu CJ, Hammond JM. Concomitant effects of growth hormone on secretion of insulin-like growth factor I and progesterone by cultured porcine granulosa cells. *Endocrinology.* 1987;121(4):1343–8.
77. Yoshimura Y, Ando M, Nagamatsu S, Iwashita M, Adachi T, Sueoka K, et al. Effects of insulin-like growth factor-I on follicle growth, oocyte maturation, and ovarian steroidogenesis and plasminogen activator activity in the rabbit. *Biol Reprod.* 1996;55(1):152–60.
78. Adashi EY, Resnick CE, D'Ercole AJ, Svoboda ME, Van Wyk JJ. Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocr Rev.* 1985;6(3):400–20.
79. Erickson GF, Garzo VG, Magoffin DA. Insulin-like growth factor-i regulates aromatase activity in human granulosa and granulosa luteal cells. *J Clin Endocrinol Metab.* 1989;69(4):716–24.
80. Hull KL, Harvey S. Growth hormone and reproduction: A review of endocrine and autocrine/paracrine interactions. *Int J Endocrinol.* 2014;2014:234014.
81. Homburg R, Eshel A, Abdalla HI, Jacobs HS. Growth hormone facilitates ovulation induction by gonadotrophins. *Clin Endocrinol (Oxf).* 1988;29(1):113–7.
82. Ibrahim ZH, Matson PL, Buck P, Lieberman BA. The use of biosynthetic human growth hormone to augment ovulation induction with buserelin acetate/human menopausal gonadotropin in women with a poor ovarian response. *Fertil Steril.* 1991;55(1):202–4.
83. Owen EJ, Shoham Z, Mason BA, Ostergaard H, Jacobs HS. Cotreatment with growth hormone, after pituitary suppression, for ovarian stimulation in in vitro fertilization: A randomized, double-blind, placebo-control trial. *Fertil Steril.* 1991;56(6):1104–10.
84. Bergh C, Hillensjö T, Wiklund M, Nilsson L, Borg G, Hamberger L. Adjuvant growth hormone treatment during in vitro fertilization: A randomized, placebo-controlled study. *Fertil Steril.* 1994;62(1):113–20.
85. Kolibianakis EM, Venetis CA, Diedrich K, Tarlatzis BC, Griesinger G. Addition of growth hormone to gonadotrophins in ovarian stimulation of poor responders treated by in-vitro fertilization: A systematic review and meta-analysis. *Hum Reprod Update.* 2009;15(6):613–22.
86. Choe SA, Kim MJ, Lee HJ, Kim J, Chang EM, Kim JW, et al. Increased proportion of mature oocytes with sustained-release growth hormone treatment in poor responders: A prospective randomized controlled study. *Arch Gynecol Obstet.* 2018;297(3):791–6.
87. Safdar L, Aghahosseini M, Alyasin A, Samaei Nourooz A, Rashidi S, Shabani Nashtaei M, et al. Growth hormone (GH) improvement of ovarian responses and pregnancy outcome in poor ovarian responders: A randomized study. *Asian Pac J Cancer Prev.* 2019;20(7):2033–7.
88. Li J, Chen Q, Wang J, Huang G, Ye H. Does growth hormone supplementation improve oocyte competence and IVF outcomes in patients with poor embryonic development? A randomized controlled trial. *BMC Pregnancy Childbirth.* 2020;20(1):310.
89. Norman RJ, Alvino H, Hull LM, Mol BW, Hart RJ, Kelly TL, et al. Human growth hormone for poor responders: A randomized placebo-controlled trial provides no evidence for improved live birth rate. *Reprod Biomed Online.* 2019;38(6):908–15.
90. Sood A, Mohiyiddeen G, Ahmad G, Fitzgerald C, Watson A, Mohiyiddeen L. Growth hormone for in vitro fertilisation (IVF). *Cochrane Database Syst Rev.* 2021;11:CD000099.
91. Suikkari A, MacLachlan V, Koistinen R, Seppälä M, Healy D. Double-blind placebo controlled study: Human biosynthetic growth hormone for assisted reproductive technology. *Fertil Steril.* 1996;65(4):800–5.
92. te Velde ER, Scheffer GJ, Dorland M, Broekmans FJ, Fauser BC. Developmental and endocrine aspects of normal ovarian aging. *Mol Cell Endocrinol.* 1998;145(1–2):67–73.
93. Dolmans MM, Demlyle D, Martinez-Madrid B, Donnez J. Efficacy of in vitro fertilization after chemotherapy. *Fertil Steril.* 2005;83(4):897–901.
94. Goud PT, Goud AP, Joshi N, Puscheck E, Diamond MP, Abu-Soud HM. Dynamics of nitric oxide, altered follicular microenvironment, and oocyte quality in women with endometriosis. *Fertil Steril.* 2014;102(1):151–9 e5.
95. Del Maestro RF. An approach to free radicals in medicine and biology. *Acta Physiol Scand Suppl.* 1980;492:153–68.
96. Sugino N. Reactive oxygen species in ovarian physiology. *Reprod Med Biol.* 2005;4(1):31–44.
97. Liu L, Trimarchi JR, Keefe DL. Involvement of mitochondria in oxidative stress-induced cell death in mouse zygotes. *Biol Reprod.* 2000;62(6):1745–53.
98. Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: Role of cumulus cells. *Biol Reprod.* 2000;63(3):805–10.
99. Huang B, Li Z, Ai J, Zhu L, Li Y, Jin L, et al. Antioxidant capacity of follicular fluid from patients undergoing in vitro fertilization. *Int J Clin Exp Pathol.* 2014;7(5):2273–82.
100. Palini S, Benedetti S, Tagliamonte MC, De Stefani S, Primiterra M, Polli V, et al. Influence of ovarian stimulation for IVF/ICSI on the antioxidant defence system and relationship to outcome. *Reprod Biomed Online.* 2014;29(1):65–71.
101. Showell MG, Mackenzie-Proctor R, Jordan V, Hart RJ. Antioxidants for female subfertility. *Cochrane Database Syst Rev.* 2020;8: Cd007807.
102. Ben-Meir A, Yahalom S, Moshe B, Shufaro Y, Reubinoff B, Saada A. Coenzyme q-dependent mitochondrial respiratory chain activity in granulosa cells is reduced with aging. *Fertil Steril.* 2015;104(3):724–7.
103. Zhang M, ShiYang X, Zhang Y, Miao Y, Chen Y, Cui Z, et al. Coenzyme Q10 ameliorates the quality of postovulatory aged oocytes by suppressing DNA damage and apoptosis. *Free Radic Biol Med.* 2019;143:84–94.
104. Bentov Y, Hannam T, Jurisicova A, Esfandiari N, Casper RF. Coenzyme Q10 supplementation and oocyte aneuploidy in women undergoing IVF-ICSI treatment. *Clin Med Insights Reprod Health.* 2014;8:31–6.
105. Caballero T, Fiameni F, Valcarcel A, Buzzi J. Dietary supplementation with coenzyme Q10 in poor responder patients undergoing IVF-ICSI treatment. *Fertil Steril.* 2016;106(3):e58.

106. Xu Y, Nisenblat V, Lu C, Li R, Qiao J, Zhen X, et al. Pretreatment with coenzyme Q10 improves ovarian response and embryo quality in low-prognosis young women with decreased ovarian reserve: A randomized controlled trial. *Reprod Biol Endocrinol.* 2018;16(1):29.
107. Hsing AW, Meyer TE, Niwa S, Quraishi SM, Chu LW. Measuring serum melatonin in epidemiologic studies. *Cancer Epidemiol Biomarkers Prev.* 2010;19(4):932–7.
108. Fernando S, Rombauts L. Melatonin: Shedding light on infertility?—A review of the recent literature. *J Ovarian Res.* 2014;7:98.
109. Tamura H, Jozaki M, Tanabe M, Shirafuta Y, Mihara Y, Shinagawa M, et al. Importance of melatonin in assisted reproductive technology and ovarian aging. *Int J Mol Sci.* 2020;21(3):1135.
110. Yie SM, Niles LP, Younglai EV. Melatonin receptors on human granulosa cell membranes. *J Clin Endocrinol Metab.* 1995;80(5):1747–9.
111. Woo MM, Tai CJ, Kang SK, Nathwani PS, Pang SF, Leung PC. Direct action of melatonin in human granulosa-luteal cells. *J Clin Endocrinol Metab.* 2001;86(10):4789–97.
112. Tamura H, Nakamura Y, Korkmaz A, Manchester LC, Tan DX, Sugino N, et al. Melatonin and the ovary: Physiological and pathophysiological implications. *Fertil Steril.* 2009;92(1):328–43.
113. Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, et al. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J Pineal Res.* 2008;44(3):280–7.
114. Eryilmaz OG, Devran A, Sarikaya E, Aksakal FN, Mollamahmutoglu L, Cicek N. Melatonin improves the oocyte and the embryo in IVF patients with sleep disturbances, but does not improve the sleeping problems. *J Assist Reprod Genet.* 2011;28(9):815–20.
115. Batioglu AS, Sahin U, Gürlek B, Oztürk N, Unsal E. The efficacy of melatonin administration on oocyte quality. *Gynecol Endocrinol.* 2012;28(2):91–3.
116. Fernando S, Wallace EM, Vollenhoven B, Lolatgis N, Hope N, Wong M, et al. Melatonin in assisted reproductive technology: A pilot double-blind randomized placebo-controlled clinical trial. *Front Endocrinol (Lausanne).* 2018;9:545.
117. Jahromi BN, Sadeghi S, Alipour S, Parsanezhad ME, Alamdarloo SM. Effect of melatonin on the outcome of assisted reproductive technique cycles in women with diminished ovarian reserve: A double-blinded randomized clinical trial. *Iran J Med Sci.* 2017;42(1):73–8.
118. Budani MC, Tiboni GM. Novel insights on the role of nitric oxide in the ovary: A review of the literature. *Int J Environ Res Public Health.* 2021;18(3):980.
119. Abdulrahim B, Scotland G, Bhattacharya S, Maheshwari A. Assessing couples' preferences for fresh or frozen embryo transfer: A discrete choice experiment. *Hum Reprod.* 2021;36(11):2891–903.
120. Battaglia C, Salvatori M, Maxia N, Petraglia F, Facchinetto F, Volpe A. Adjuvant l-arginine treatment for in-vitro fertilization in poor responder patients. *Hum Reprod.* 1999;14(7):1690–7.

INNOVATIVE THERAPIES IN DIMINISHED OVARIAN RESERVE (DOR) AND PRIMARY OVARIAN INSUFFICIENCY (POI) PATIENTS

Francesc Fabregues, Janisse Ferreri, and Marta Mendez

Introduction

Unlike what happens with other organs of the human body whose functioning is maintained in a constant way until the end of life, the ovary has a finite activity conditioning not only regarding its effects on women's tissues and organs but also on their reproductive function [1].

The ovarian aging process, related to the progressive depletion of the pool of primordial follicles, already begins in intrauterine life, where the maximum is reached around 20–22 weeks of gestation with 7–8 million follicles, reducing to approximately 1 million in the moment of birth. Subsequently, follicular depletion continues, reaching a total of 400,000 follicles at menarche, clearly accelerating from the age of 35, compromising the fertility of women [2, 3].

The current concept of ovarian reserve refers to the total number of oocytes that women's ovaries have at a given time, and we currently have ultrasound and hormonal markers that allow us to define the magnitude of this aspect from a quantitative point of view. The ovarian reserve of a woman can be conditioned by two different causes; by an initial "pool" of smaller follicles already in intrauterine life and by a postnatal cause that has accelerated follicular depletion. Along these lines and with the aim of clarifying some confusing aspects in the literature, it is worth treating some concepts that are of great importance to the subject at hand [4, 5].

DOR (diminished ovarian reserve) is accepted as a situation that refers to a quantitative decrease in the pool of primordial follicles not concordant with the chronological age of the woman and that can be diagnosed by a decrease in the antral follicle count by transvaginal ultrasound and/or by low plasma levels of anti-Müllerian hormone (AMH) [6]. This issue corresponds to another concept very frequently cited in the literature, which is poor ovarian reserve or poor ovarian response (POR), which refers to a POR to hormonal stimulation in the context of an *in vitro* fertilization (IVF) cycle.

For many years, the term POF (premature ovarian failure) was used to define the situation in which ovarian activity ceases prematurely (before the age of 40). However, in recent years this concept has been abandoned and replaced by primary ovarian insufficiency (POI) since, according to most authors, it better defines an ovarian functional situation with interesting aspects that we will develop throughout this chapter. POI is defined as the clinical situation in which a woman under 40 years of age presents elevated FSH levels (>25 IU/L) in two determinations separated by four weeks and periods of oligo/amenorrhea of more than four months. This definition includes very specific clinical and analytical aspects and translates to the end of the premature "ageing" process of the ovary [7].

Currently, the most effective reproductive solution in patients with DOR and POI is the donation of oocytes. Nevertheless, in recent years innovative therapies have been tested which aim to

awake follicles that are still present in the ovaries of these patients [8].

Some surgical procedures focus on the stimulation of the ovarian AKT signalling and disrupt Hippo signalling (**conventional *in vitro* activation**) or alone Hippo signalling disruption (**drug-free IVA**); others intend to employ the growth factors contained in blood (**platelet-rich plasma**) or from the use of stem cells (**stem-cell-based therapy**) or to try mitochondrial enrichment in order to improve oocyte quality (**mitochondrial therapies**).

This chapter will analyse the physiological bases, the preliminary results obtained, and the limitations and challenges that come with the application of these techniques.

In vitro activation (IVA)

Background and physiological bases of the technique

Although the complete mechanism of follicular activation remains undeciphered, studies in knockout mice have shown that oocyte-specific deletion of the PTEN and Foxo3 gene promotes the activation and growth of all primordial follicles [9, 10]. The PTEN gene encodes a phosphatase enzyme that negatively regulates the PI3K-AKT-Foxo3 signalling cascade. The activation of dormant primordial follicles has also been promoted by using PTEN inhibitors and/or AKT activators in both murine and human ovaries [11–15].

The coordination of cell proliferation and cell death is essential for the maintenance of organ size and tissue homeostasis during postnatal life. In mammals, the coordination of both processes is orchestrated by the Salvador–Warts–Hippo (SWH) pathway. This signalling pathway consists of different negative regulators acting in a cascade of kinases that ultimately antagonizes the transcriptional coactivator yes associated protein (YAP) and its PDZ-binding motif (TAZ) inducing growth suppression [16–18].

YAP is inactivated by Hippo pathway-mediated phosphorylation, which excludes it from the nucleus, whereas loss of Hippo signalling promotes the accumulation of YAP in the nucleus and an increase in its activity. Once inside the nucleus, the YAP protein acts in coordination with TAZ transcriptional activators to trigger the expression of growth factors. This results in increased cell proliferation and growth [19–22].

Unlike most signalling pathways activated by extracellular ligands, Hippo is regulated by a network of components related to cell adhesion, shape, and polarity. These cellular characteristics are mediated by rapid changes in polymerization from globular actin (G-form) to filamentous actin (F-form) that are induced by tissue fragmentation and are the triggers for inhibition of the Hippo pathway [23, 24] (Figure 56.1).

Recent studies have shown that activation of the AKT pathway would primarily activate primordial follicles while inhibition of the Hippo pathway would essentially act at the level of secondary follicles by activating them [14, 25] (Figure 56.2).

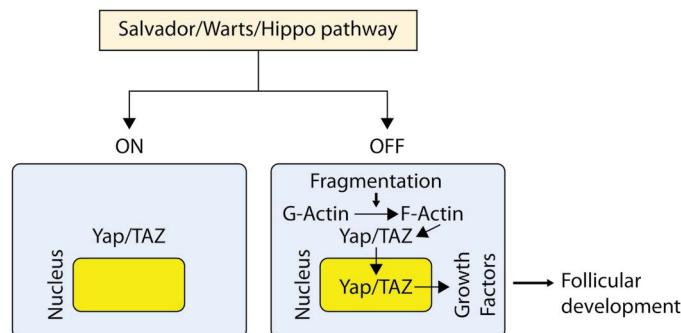


FIGURE 56.1 The Hippo pathway.

In 2013, based on data obtained from animal experimentation, Kawamura et al. [14] regarding the factors related to the activating and inhibitory pathways of the early phases of folliculogenesis, published the results of a technique named *in vitro* activation (IVA). In POI patients, a laparoscopy was performed, removing an ovary and then releasing the cortex and fragmenting it into 1×1 mm pieces. These fragments were incubated for 48 hours with AKT (phosphatidylinositol 3-kinase) stimulating substances such as 740YP and with PTEN (phosphatase and tensin homolog deleted on chromosome 10) inhibitors such as bpV (HOpic). Fragmentation of ovarian tissue has been linked to inactivation of the Hippo pathway, whose mechanisms of action have already been discussed at the beginning of this chapter. In a second laparoscopy these ovarian fragments were implanted in the tubal serosa of the contralateral adnexa. Ovarian follicular activations, oocytes, and new-borns have been obtained with this technique [14, 25–27].

Recently, a simplification of conventional IVA has been published, named drug-free IVA, avoiding chemical activation of ovarian tissue and focusing exclusively on the tissue fragmentation

being reinserted into the contralateral ovary and/or adjacent peritoneum [28–34]. This technique is performed by a single laparoscopy and follicular activation that has also been reported in 50% of POI patients achieving mature oocytes, embryos, and live new-borns [29] (Figure 56.3).

As it has been previously mentioned, activation of the AKT pathway would be effective in the activation of primordial follicles, whereas inhibition of the Hippo pathway would act mainly at the level of secondary follicles, i.e. at more advanced stages of folliculogenesis. In this sense, it has been suggested that conventional IVA could be used in patients with long-duration POI, whereas drug-free IVA would be more effective in cases with POI recent or even in cases of DOR [35, 36]. It should be noted that, despite the encouraging results, the IVA and drug-free IVA techniques are considered experimental and are not yet routinely applicable in POI patients.

Results

Table 56.1 summarizes the results obtained with IVA and drug-free IVA up to the time of this publication. Conventional IVA has

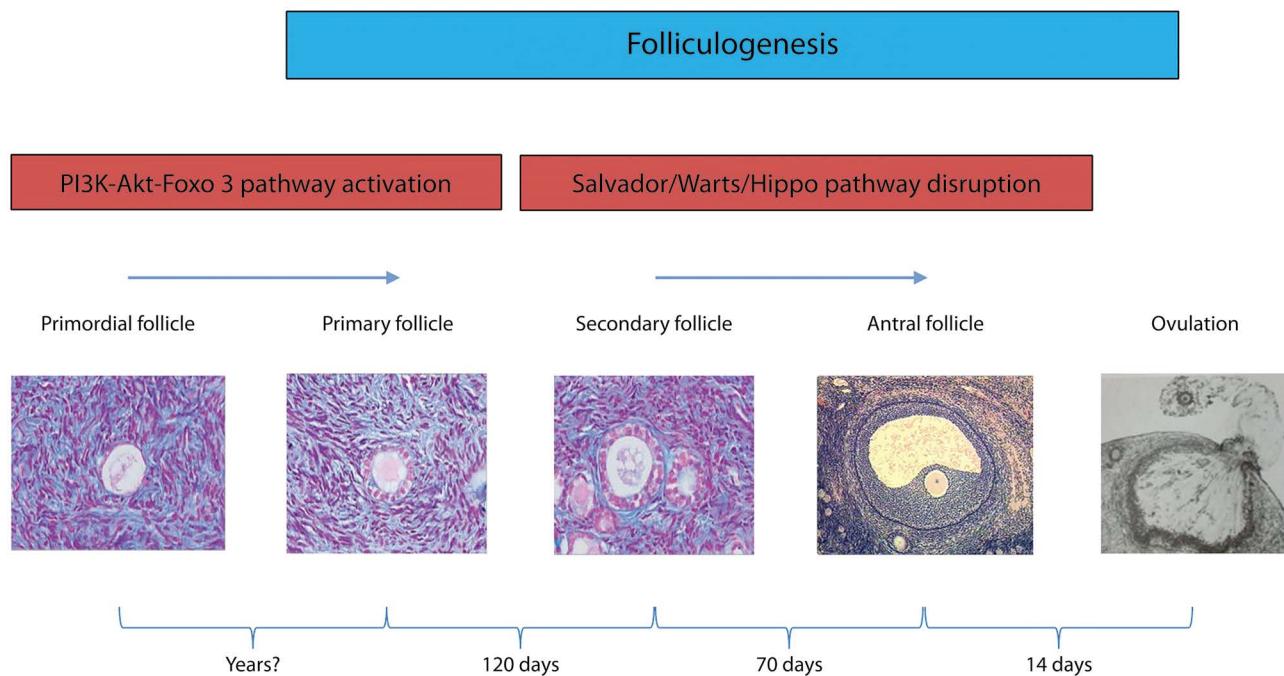


FIGURE 56.2 Folliculogenesis.

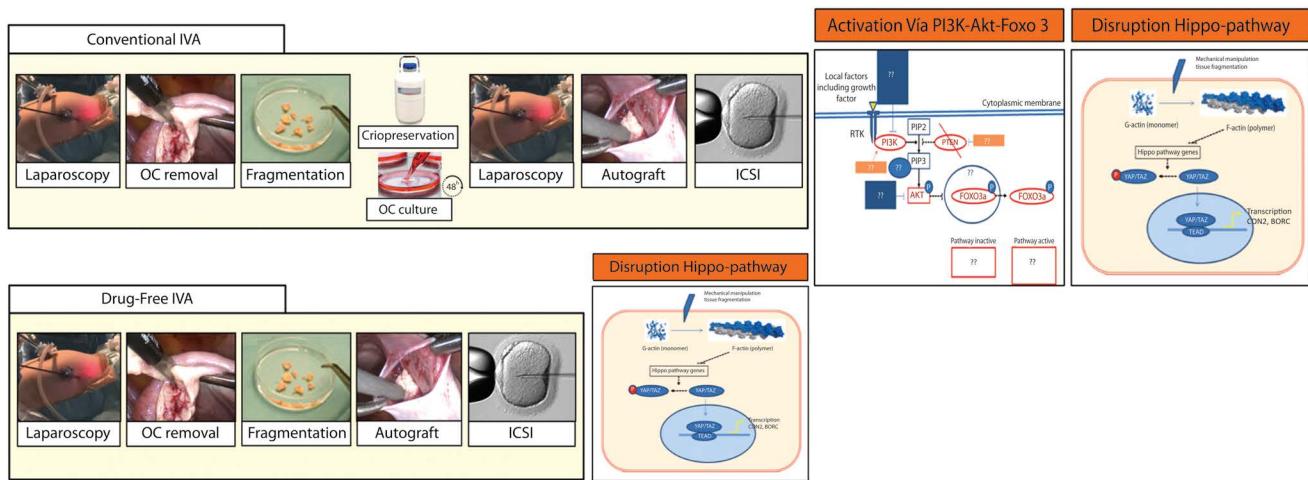


FIGURE 56.3 Conventional and drug-free IVA. (From [29] with permission.)

been tested only in POI patients, but drug-free IVA has been used in both POI patients and POR patients. As can be seen, mainly in terms of live births, the results are encouraging. However, the findings are more confusing in POR patients, as it is difficult to analyse the direct effect of the technique in terms of the ability to recover ovarian activity. Lunding et al. [33] published a prospective cohort study of 20 women (mean age 37.4) with POR diagnosed by the Bologna criteria. These women underwent unilateral ovarian biopsy followed by tissue fragmentation and autotransplantation under the peritoneal serosa beneath the right ovary. The contralateral unbiopsied ovary served as control in each patient. Following the surgery, patients were monitored for 10 weeks by antral follicle count (AFC) and ovarian volume, as well as levels of AMH, FSH, luteinizing hormone (LH), oestrogen, progesterone, and other markers. At 10 weeks, all patients underwent COS for IVF/ICSI, after which a day 2 fresh embryo transfer was performed. If the patients were not pregnant, monitoring

continued monthly for one year and further ART was recommended as appropriate. Comparison of follicle development at 10 weeks revealed no difference between the biopsied and the control ovaries. AFC increased steadily after treatment, but interestingly the biopsied ovary had a significantly lower AFC than the control. Twelve of 20 patients (60%) achieved pregnancy, three of them spontaneously before undergoing COS. Authors concluded that the study did not indicate that biopsying, fragmenting, and autotransplanting ovarian cortical tissue increases the number of recruitable follicles for IVF/ICSI after 10 weeks. However, the authors suggest that the pregnancies achieved could be explained by a more long-term effect of the technique. Recently, Kawamura et al. analysed the drug-free IVA effect in POR patients confirming its effectiveness [30].

Lastly, a recent meta-analysis which included eight studies concluded that drug-free activation of ovarian tissue in comparison with drug-included activation seemed to be more efficient [37].

TABLE 56.1 Human Studies Involving IVA and Drug-Free IVA in POI and POR Patients

Study (Ref.)	Procedure Type	No. Patients	Inclusion Criteria	Resumption Ovarian Activity (n; %)	Clinical Pregnancy Rate (n; %)	Live Birth Rate (n; %)
Kawamura et al. 2013 [14], Suzuki et al. 2015 [26]	IVA	37	POI	9 (24.39)	3 (8.1)	2 (5.4)
Zhai et al. 2016 [27]	IVA	14	POI	6 (42.8)	1 (7.1)	1 (7.1)
Pellicer et al. 2017 ^a	OFFA (Drug-Free IVA)	14	POI		3 (21.4)	3 (21.4)
Zhang et al. 2019 [32]	Biopsy/Scratch	80	POI	11 (13.7)	1 (1.2)	1 (1.2)
Lunding et al. 2019 [33]	Drug-Free IVA	20	POR		12 (60)	10 (50)
Fabregues et al. 2018 [28] Ferreri et al. 2020 [29]	Drug-Free IVA	14	POI	7 (50)	4 (28.5)	4 (28.5)
Manhajan et al. 2019 [31]	Drug-Free IVA	1	POI	1 (100)	-	-
Kawamura et al. 2020 [30]	Drug-Free IVA	11	POR	9 (81.8) ^b	5 (45.4)	3 (27.2)
Patel et al. 2021 [34]	Drug-Free IVA	1	POR	1	1 (33.3) ^c	

Notes:

^a Unofficial data are from conference presentations of stated scientist.

^b Patients increased antral follicle count.

^c Miscarriage 9 weeks. Spontaneous pregnancy 3 months after miscarriage. 24 weeks pregnancy at the time of publication

OFFA: Ovarian fragmentation for follicular activation.

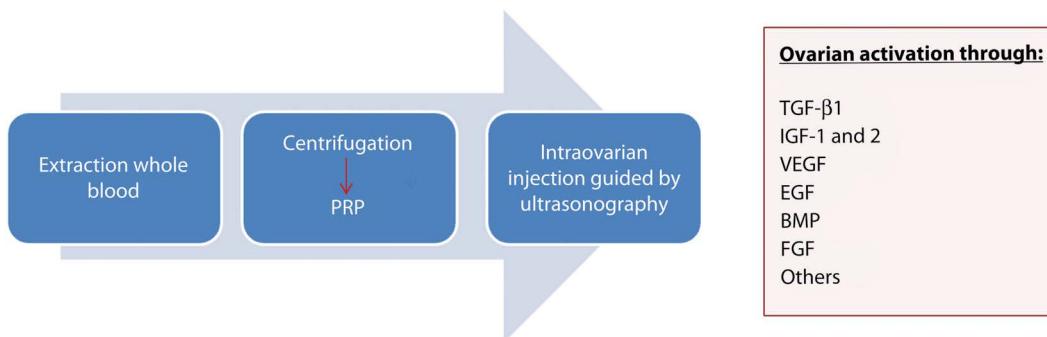


FIGURE 56.4 Ovarian activation via the PRP technique.

Limitations and challenges

Despite the promising results obtained with surgical techniques for ovarian activation, there are still controversial aspects that must be solved.

Firstly, in any published study there's a control group of patients compared with those who have received treatment. Secondly, predictive criteria for response have not yet been established. Although it has been suggested that drug-free IVA could be more useful in recent POI, there are no conclusive data. Third, clinical criteria for patient follow-up should be better established. The length of follow-up, and especially whether to opt for spontaneous gestation or to always resort to IVF of the oocytes obtained.

IVA and drug-free IVA have in common their action on the Hippo signalling pathway, which is a key factor in the mechanisms of mechanotransduction [22]. Mechanobiology is a field of biology that relates physico-mechanical changes to cell function and gene expression [38, 39]. In this sense, and taking into account many studies that have related folliculogenesis and follicular distribution in the ovary, we can suggest that future studies should focus on the relationship between the characteristics of the extracellular matrix and the role that its different components may play in physiological ovarian aging and in cases where this may be accelerated in a pathological way as in POI and POR patients [24, 39, 40].

Platelet-rich plasma

Background and physiological bases of the technique

Platelet-rich plasma (PRP) is obtained from centrifuged peripheral blood by different methods. The content is 80%–90% platelets, with a low content of leukocytes and red blood cells.

Many studies have provided information on its regenerative efficacy in different tissues, having been used in musculo-skeletal, maxillo-facial, and dental pathology [8, 41]. PRP is now starting to become an area of interest in reproductive medicine, more specifically focusing on infertility. POR, menopause, POI, and thin endometrium have been the main areas of research [42]. It has been suggested that the regenerative properties of PRP can be explained by higher concentrations of growth factors such as transforming growth factor-β (TGF-β), insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and other substances related in some way to folliculogenesis [43, 44].

Several experimental studies have linked factors released by platelets to ovarian function. Some factors are involved in follicular activation, such as bone morphogenic protein (BMP) and

TGF-β-1 [45]; others with the regulation of ovarian angiogenesis such as VEGF [46] and also with follicular maturation and granulosa cell proliferation such as fibroblast growth factor (FGF) [47].

The technique consists in the administration of 2–4 mL of PRP through an injection with a 16–18 G needle under transvaginal ultrasound and sedation. The PRP is deposited in the ovarian cortex superficially and in the subcortical area (Figure 56.4).

Studies have reported that its efficacy has been analysed in two aspects: on one hand, in the changes observed in ovarian reserve markers and in the results of IVF cycles in patients with previous POR, and on the other hand, in the possibility of achieving reactivation of ovarian function in patients diagnosed with POI.

Since it is a simple technique, a large number of studies have been reported in recent years that have evaluated its possible efficacy in both POI and POR patients.

Despite the plausibility of its efficacy, depending on the different factors released by suitably activated platelets, the preliminary results obtained need to be confirmed.

Results

Table 56.2 summarizes the results obtained with PRP in POI and POR patients up to the time of this publication. In general, the results obtained with PRP infusion in POR patients show encouraging findings in terms of improved ovarian reserve markers and response [47–52]. However, the only prospective controlled study did not show a significant improvement in live birth rate [52].

Regarding the results obtained in POI patients, the experience is less. However, 22 live births have been reported up to the time of this publication [48, 53–56].

No complications have been reported in any of the published studies and therefore it seems that we are facing a therapeutic option in which future randomized studies should provide definitive information.

Limitations and challenges

PRP is a term used to describe a fraction of the blood after processing. The platelet “activation” method is essential for PRP to exert its regenerative potential. When we analyse the different studies published on the effect of PRP in infertile patients, we can observe that some studies describe using calcium [48, 49, 52], or thrombin [57], while others inject quiescent platelets or simply do not state their activation status [50, 55]. It is important because the presence of leukocytes and erythrocytes, depending on the method used for activation, can lead to the presence of substances with the opposite effect to that desired.

Interestingly, platelets release significant quantities of IL-15 when activated [58]. Increased IL-15 concentrations in follicular

TABLE 56.2 Human Studies Involving PRP in POI and POR Patients

Study (Ref)	Study Design	Inclusion Criteria	No. of Patients in Which PRP Was Used	Findings	Live Birth Rate (n; %)
Sfakianoudis et al. 2018 [53]	Case report	POI	1	Natural IVF cycle-1 oocyte and one embryo transfer	
Sfakianoudis et al. 2020 [48]	Case series	POI and POR	30 POI 30 POR	POI: Ovarian follicular resumption : 18/30 POR: Improve IVF performance: 21 ET	3 (10) in POI 12 (40) in POR
Sills et al. 2018 [49]	Case series	POR	4		1 (25)
Pantos et al. 2019 [54]	Case series	POI	3	All patients resumed menstruation	1 (33.3)
Farimani et al. 2019 [50]	Case series	POR	12	The oocyte yield and the average number of retrieved oocytes and resulting embryos was higher after PRP treatment	3 (25)
Cakiroglu et al. 2020 [55]	Case series	POI	311	23 spontaneous pregnancies with 16 LB 57 ET with 9 LB 25 patients with cryopreserved embryos	25 (8.03)
Hsu et al. 2020 [56]	Case report	POI	1	PRP and FSH/LH intraovarian injection. IVF-ET and Twins	2 (100)
Melo et al. 2020 [52]	Prospective controlled study	POR	46 treated with PRP 37 controls	Biochemical and clinical pregnancy rates higher in PRP group. No differences in LB	5 (11.1)
Pacu et al. 2021 [51]	Case series	POR	20	Cancellation rate decreased following PRP treatment while the number of collected oocytes, number of oocytes in metaphase II rose	3 (15)

fluid have been negatively correlated with pregnancy outcomes in IVF, indicating that this cytokine may be detrimental to follicle maturation [59].

This interplay and opposing effects of PRP constituents in different contexts serve to illustrate the importance of detailed studies of the mechanisms of how PRP might act on the ovary, and much additional work is required before any conclusions can safely be drawn.

Another important aspect using PRP is the lack of homogeneity in the protocols applied. Differences in the amount and site of infusion, as well as in the follow-up of the patients represent a limitation of this approach.

Although in most studies, the volume of plasma injected into the ovary is 2–4 mL, the dose required for optimal effect is not specified. Moreover, in some cases, the injection is unilateral and in others bilateral. There is also some controversy as to the exact site of injection. Some authors specify that it should be performed in the ovarian cortex, while most do not. On the other hand, the exact follow-up period that should be performed on patients after PRP infusion is also unclear. In this regard, it should be noted that recent studies show that the possible beneficial effect would occur in the first months of PRP administration, since its effect would cease after six months [50].

Stem-cell-based therapy

Background and physiological bases of the technique

One of the most controversial issues in the field of human reproduction is the presence of oogonia stem cells (OSCs) in the adult ovary. While some authors claim to have identified them [60, 61], others have not confirmed it, and recently it seems that this possibility could be discarded [62, 63].

However, there is an agreement regarding the existence of a stem cell (SC) niche in the ovary, as it has also been demonstrated in the hemopoietic, gastrointestinal, and neuronal systems [64, 65]. SC niche refers to the micro-environment surrounding SCs, and it has recently been shown that ovarian aging is related to the aging of the niche [66]. Following this concept, one of the most promising strategies pursues the regeneration of ovarian niche using SCs in order to promote development of remaining follicles within the ovary [44, 67].

Over the last years, several experimental studies conducted in animal models of POI have suggested the benefit of SC-based therapy in the resumption of ovarian function. Most of these studies were performed in rodents with chemotherapy-induced POI, and the results demonstrated an improvement in ovarian function and higher pregnancy and live birth rates than controls [68–70]. SCs from different sources have been tested, but the most promising results were obtained with SCs of mesenchymal origin (MSCs), which can notably be found in amniotic fluid, menstrual blood, the umbilical cord [71, 72], bone marrow [72, 73–76], and adipose tissue [77].

Pilot studies and clinical trials in POI patients have been performed with bone marrow-derived SCs (BMDSCs). The possibility of obtaining a large number of cells, from an autologous source, by means of well-established protocols, makes them a valuable candidate.

The mechanism of action of MSCs is very complex and still under investigation; however, three potential ways by which they could act on the ovary have been considered [43, 66]. Firstly, it would be through potential differentiation, which would mean their capacity to transform into different cell types and consequently replace those damaged or absent in the target tissue. Secondly, it would be through what is currently known as the “homing” phenomenon, i.e. their capacity to populate defective

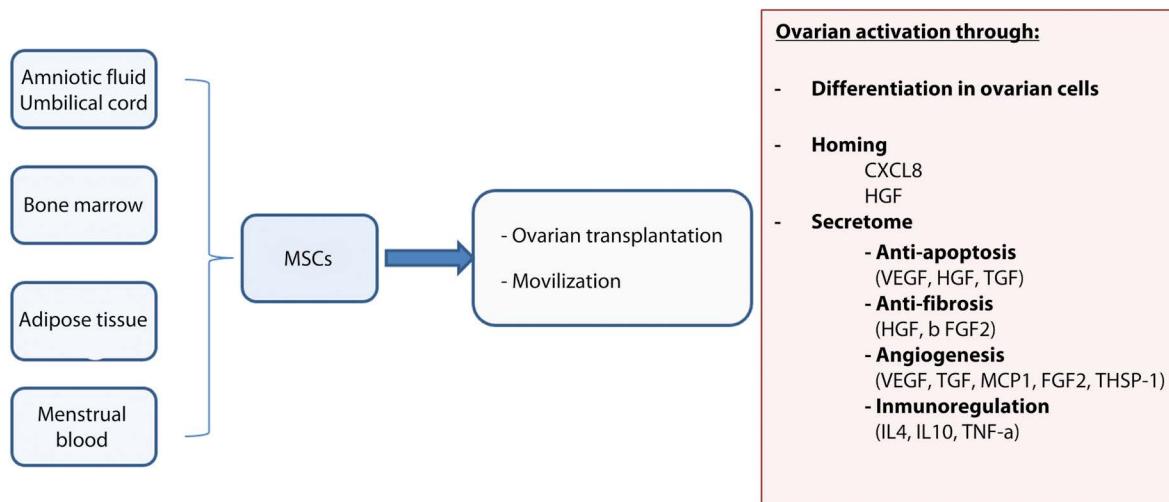


FIGURE 56.5 Ovarian activation via the secretome.

tissues or organs. Finally, through the secretome, which consists of the release of cytokines and growth factors that play an essential role in promoting angiogenesis, inhibiting apoptosis and fibrosis, and modulating the immune response (Figure 56.5).

Results

Table 56.3 shows the preliminary results with SC-based therapy in the context of POR and POI patients.

Although there are differences in the source of SCs and in the method of administration, up to now most experience is focused on the use of bone marrow-derived cells and basically on their local administration by laparoscopy [73–75], ovarian puncture under ultrasound control [71, 72, 77], or intraarterial catheter [70, 76].

The technology has been applied mainly in POI patients with resumption rates of ovarian activity between 50% and 80%, both in terms of ovulatory cycles and in the improvement of ovarian reserve markers.

Unlike the other innovative therapies just discussed, the number of live births is lower, probably because the existing knowledge about this technique is also scarcer.

Of note is the study by Pellicer et al. [76] in which the effect of SC mobilization after administration of GSC-F, without the need for invasive methods, was analysed for the first time, in which an increase in AFC was observed in 50% of patients with POI. These results would be based on studies of the same group in which it has been suggested that factors such as FGF-2 and THSP-1 released by mobilized SCs would be able to activate the ovarian niche and therefore awaken dormant follicles still existing in the ovary of POI patients. While awaiting definitive results, this seems to be a strategy for the future [67, 70].

Limitations and challenges

Although the use of MSCs has been widely explored in animal studies, there are considerations to be made. Most animal experiments have been performed by eliciting POI with cytostatic agents that do not necessarily simulate POI in humans. On the other hand, differences in the immune system can affect the immunogenicity of transplanted cells, which may elicit autoimmune responses not evident in animals. In this sense, the majority

of work in this area has been largely preliminary, observational, and uncontrolled; thus with the well-established possibility of unexpected ovulation and pregnancy, many such studies require extreme caution in interpretation.

Despite all of the preceding, the current results suggest a plausibility in its efficacy. Undoubtedly, the large number of studies on the topic registered in the clinicaltrial.gov database will provide a wealth of information on the subject [44, 67].

Mitochondrial therapies

Background and physiological bases of the technique

Ovarian aging has connotations not only on the quantity of oocytes but also on their quality. Embryonic aneuploidy is closely related to impaired capability of old oocytes to organize microtubules during spindle assembly, one of the most energy-consuming steps of meiosis resumption [78]. One of the main factors related to the high probability of an error during the second meiotic division has been mitochondrial dysfunction. In fact, aged oocytes have reduced mitochondria, resulting in a low fertilization rate and poorer embryo development [79, 80].

Recently, different techniques with the aim of using different autologous and heterologous sources of mitochondria have been tried to re-establish oocyte quality in unfavourable reproductive scenarios [81, 82] (Figure 56.6).

Before discussing the different techniques of oocyte rejuvenation by mitochondrial supplementation, we must point out that these approaches will act once the first meiotic division has occurred, therefore they cannot repair any aneuploidy that originated before [83].

In the heterologous approach, the mitochondria come from an external source which is a donor oocyte. Mitochondrial enrichment can be performed in this context by relocating a healthy cytoplasm into the patient's oocyte (partial cytoplasm transfer) [84–87] or replacing the compromised cytoplasm with a competent one by means of nuclear transfer technology (total cytoplasm transfer) [88–89].

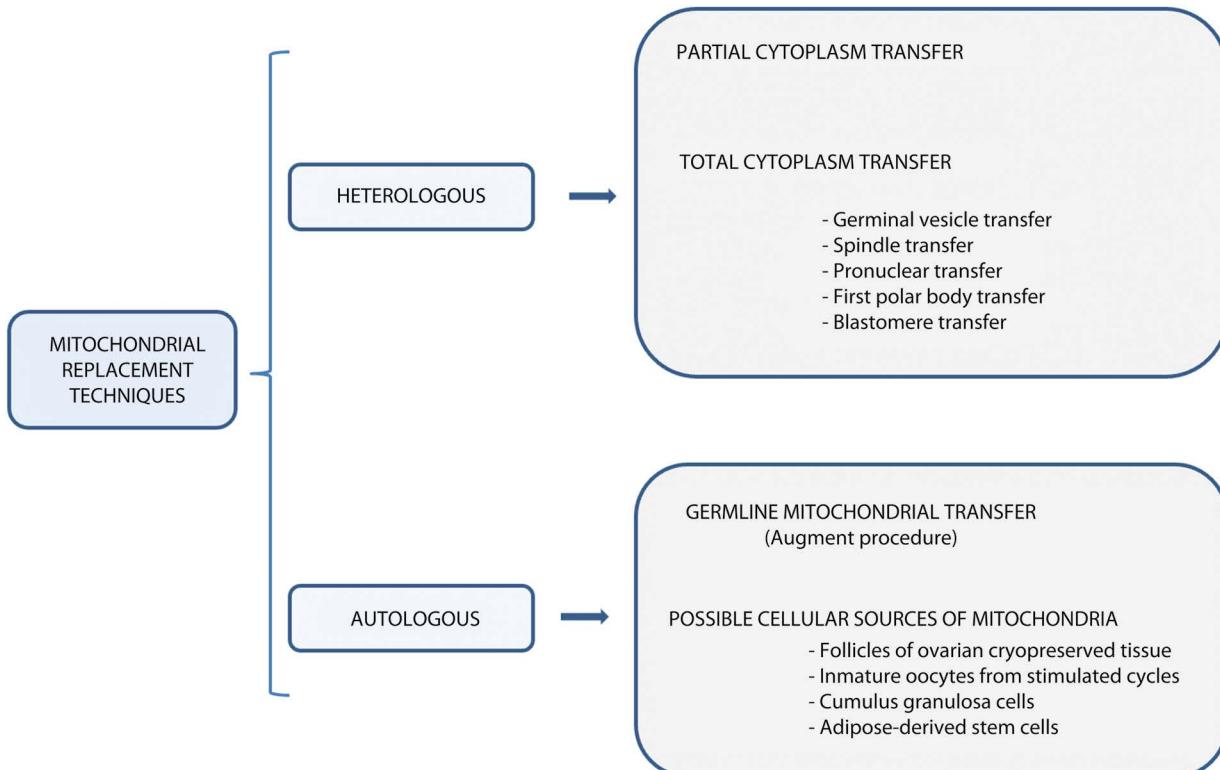
Partial cytoplasm transfer consists of the aspiration of cytoplasm from a donor oocyte and its introduction into the patient's

TABLE 56.3 Human Studies Conducted with Stem Cells (SCs) in POI and POR Patients

Study (Ref)	No. Patients	Inclusion Criteria	SC Source and Administration Method	Findings	Live Birth Rate (n; %)
Edessy et al. 2016 [73]	10	POI	BMMSCs Laparoscopic injection	2 patients with resumption ovarian function	1 (10)
Gabr et al. 2016 [74]	30	POI	BMMSCs Laparoscopic injection in one ovary and one ovarian artery	18/30 (60% showed ovulation)	1 (3.3)
Gupta et al. 2018 [75]	1	POR	BMMSCs Laparoscopic injection	AFC and AMH increased 8 weeks after	1 (100)
Herraiz et al. 2018 [70]	17	POR	BMMSCs Intraarterial catheter in ovarian artery	81.3% patients improved AFC and AMH	3 (17.6)
Ding et al. 2018 [71]	14	POI	UCMSCs Transvaginal injection by ultrasonography in one ovary	6 patients with UCMSCs and 8 with UCMSCs on collagens scaffold One pregnancy in each group- one 21-trisomy and one ongoing >20 weeks pregnancy	
Yan et al. 2020 [72]	61	POI	UCMSCs Transvaginal injection by ultrasonography in both ovaries	3 pregnancies with IVF and 1 spontaneous	4 (6.7)
Pellicer et al. 2020 ^a [76]	10	POI	BMMSCs Ovarian artery by intraarterial catheter (ASCOT) and SC mobilization (G-CSF). Randomization	AFC increased in 50% patients with G-CSF and 66% in ASCOT group ASCOT arm- 1 ongoing pregnancy	
Mashayekhi et al. 2021 [77]	9	POI	ADSCs Transvaginal injection by ultrasonography in one ovary	Non-randomized controlled open-label study comparing different amount SC	

Abbreviations: BMMSCs, Bone marrow mesenchymal derived stem cells; UCMCs, umbilical cord mesenchymal stem cells; ADSC, adipose derived stromal cells; G-CSF, granulocyte colony stimulating factor.

^a Preliminary data from interim analysis reported.

**FIGURE 56.6** Mitochondrial replacement techniques.

oocyte, providing components of the young oocyte capable of improving its viability. Subsequently, the rejuvenated oocyte is fertilized by ICSI. After the first pregnancy reported by Cohen et al. in 1997 [84] and some encouraging results in preliminary studies, the technique has been the subject of great controversy. In 2001, the Food and Drug Administration (FDA) suspended its use owing to ethical and technical concerns [90], as the introduction of foreign cytoplasm leads to mitochondrial heteroplasmy in the patient's oocyte. It has been suggested that the presence of the third genetic material (mtDNA from the donor) could interfere with the close communication between the nuclear and mitochondrial DNA from the recipient and may lead to unpredictable consequences for not only the developing embryo but also for the offspring's subsequent long-term health [91, 92].

In order to reduce the risk of heteroplasmy, other techniques have been proposed in which the amount of material transferred into the host oocyte is reduced.

Germinal vesicle (GV), spindle, pronuclear, polar body, and blastomere transfer constitute different ways of relocating the genetic material from a patient's compromised oocyte or zygote to a healthy cytoplasm [81, 82].

Although all these techniques have been explored in animal experiments, there is little information on their application in daily clinical practice. The different types of total cytoplasmic transfer have been proposed to overcome maternal mitochondrial disease transmission by transferring the maternal spindle into a healthy recipient donor cytoplasm (the "three-parent baby" technique).

However, both ethical and technical aspects have been a source of controversy. The problem of heteroplasmy is still present and therefore its clinical application should still be viewed with scepticism.

With the aim of solving concerns raised by the use of heterologous donor mitochondria, an autologous approach has been reported. The existence of germline SCs in the adult mammalian ovary of both mice and humans has been reported. Although their potential contribution to postnatal oogenesis remains questionable, when isolated, these ovarian SCs constitute an autologous source of high-quality germline mitochondria from the same cell lineage [93].

Based on these facts, the augment technique has been proposed [94]. Briefly, ovarian cortex is obtained by laparoscopy and by using specific antibodies, mitochondria from OSCs are

TABLE 56.4 Human Studies Involving Mitochondrial Replacement Techniques

Study (Ref)	No. Patients	Diagnosis	Method	Findings	Outcomes
Cohen et al. 1997 [84]	1	Inadequate embryo development in 4 IVF cycles	Partial cytoplasm transfer (Heterologous approach)	9 of 14 patient eggs showed fertilization. 6 embryos with normal morphology. 4 were transferred	1 live birth
Cohen et al. 1998 [85]	7	POR and Repeated Implantation failure	Partial cytoplasm transfer (Heterologous approach)	Normal fertilization was significantly higher after injection of ooplasm (62%) vs electrofusion (23 %)	3 pregnancies after injection 1 live birth, 1 miscarriage and 1 pregnancy ongoing
Huang et al. 1999 [86]	9	Repeated implantation failure	Partial cytoplasm transfer of trypsonucleate zygotes (Heterologous approach)	62 metaphase II oocytes were injected—39 (62%) had correct embryo cleavage	5 live births
Dale et al. 2001 [87]	1	Unfavourable embryo cleavage in previous IVF cycles	Partial cytoplasm transfer (Heterologous approach)	Good embryo cleavage in 6 oocytes after ooplasm injection—4 embryos were transferred	Twin pregnancy—2 live births
Tanaka et al. 2009 [88]	-	-	Total cytoplasm transfer (Spindle transfer) (Heterologous approach)	25 oocyte reconstructed developed 7 (28%) blastocyst stage. 98 oocyte control developed 3 blastocyst stage (3.1%)	
Zhang et al. 2017 [89]	1	Leigh syndrome	Total cytoplasm transfer (Spindle transfer) (Heterologous approach)	5 oocytes reconstituted—4 fertilized by ICSI 1 euploid blastocyst transferred	1 live birth
Fakih et al. 2015 [95]	25	Repeated implantation failure and unfavourable embryo cleavage in previous IVF cycles	AUGMENT (Autologous approach)	14 embryo transfers in Augment group vs 2 embryo transfers in ICSI-only group	8 pregnancies ongoing
Oktay et al. 2015 [96]	10	Unfavourable embryo cleavage in previous IVF cycles	AUGMENT (Autologous approach)	Improve fertilization rate and embryo cleavage	4 live births
Labarta et al. 2019 [96]	57	Unsuccessful previous IVF and Unfavourable embryo cleavage	AUGMENT (Autologous approach)	Randomization : Control group: 250 MII oocytes Augment group: 253 MII oocytes. Blastocyst formation rate worse in Augment group	3 live births in Augment group.

obtained from the cortex. During ICSI mitochondrial suspension is injected along with the spermatozoon.

Although some preliminary studies have reported improved pregnancy rates in poor prognosis patients undergoing IVF [95, 96], a triple-blind, randomized, single-centre, controlled experimental study not only failed to demonstrate the beneficial effects of the technique, but the interim analysis stopped patient recruitment, considering the invasive character and the cost of the technique [97].

Results

Table 56.4 summarizes the experience with the different mitochondrial replacement techniques, the most relevant findings, and their preliminary results.

Although a heterologous approach using donor oocyte cytoplasmic material was initially considered, the risk of heteroplasmy mentioned earlier and the reluctance of the scientific societies put the subject on hold until an autologous approach could be considered.

Augment technique was proposed as a novel tool to improve embryo quality in humans and some studies evaluate its efficacy. In a prospective cohort and descriptive analysis, Fakih et al. [95] published results from 59 patients in two clinics (United Arab Emirates and Canada). Authors reported marked improvement of pregnancy rates compared to previous IVF cycles and improvements in embryo cleavage after augment with eight ongoing pregnancies. Similarly, Oktay et al. [96] published four live births in 10 patients who completed the augment technique.

Contrary to previous studies, Labarta et al. [97], in a prospective randomized study, did not confirm the previous results. In a total of 56 patients in whom oocyte retrieval was performed, 253 MII oocytes were inseminated in the augment group and 250 in the control group. Fertilization rates were similar, but blastocyst formation rate per zygote was higher in the control group (41.1% vs 23.3%; $p = 0.0001$). Euploid rate per biopsied blastocyst and per MII oocyte were similar, and, finally, cumulative live birth rates per transferred embryo were similar.

According to these findings, the author concludes that augment does not seem to improve prognosis and is not a feasible treatment to improve embryo quality.

Limitations and challenges

Unlike the innovative techniques discussed in this chapter that aim at activating dormant follicles in the clinical scenarios of POI and POR, mitochondrial replacement techniques have as their main target the improvement of oocyte quality and thus a better outcome in embryo quality.

The results reported for both heterologous and autologous approaches have not confirmed the hopes that were initially placed in them. Aspects related to safety and, above all, methodological deficits in the studies carried out have not allowed them to be consolidated for daily use in clinical practice.

In spite of this, new sources for obtaining mitochondria are being investigated, such as immature oocytes from stimulated cycles, oocytes from cryopreserved ovarian tissue, and SCs from adipose tissue, which may provide more information on the subject in the future.

References

- Faddy MJ, Gosden RG, Gougeon A, et al. Accelerated disappearance of ovarian follicles in mid-life: Implications for forecasting menopause. *Hum Reprod*. 1992;7:1342–6.
- Wallace WH, Kelsey TW. Human ovarian reserve from conception to the menopause. *PloS One*. 2010;5:e8772.
- Broekmans FJ, Soules MR, Fauser BC. Ovarian aging: Mechanisms and clinical consequences. *Endocr Rev*. 2009;30:465–93.
- Velde T, Pearson ER. The variability of female reproductive ageing. *Hum Reprod Update*. 2002;8:141–54.
- Richardson MC, Guo M, Fauser BC, et al. Environmental and developmental origins of ovarian reserve. *Hum Reprod Update*. 2014;20:353–69.
- Pastore L, Christianson M, Stelling J, et al. Reproductive ovarian testing and alphabet soup of diagnosis: DOR, POI, POR, and FOR. *J Assist Reprod Genet*. 2018;35:17–23.
- Webber L, Davies M, Anderson L, et al. ESHRE guideline: Management of women with premature ovarian insufficiency. *Hum Reprod*. 2016;31:926–37.
- Reig A, Herranz S, Pellicer A, Seli E. Emerging follicular activation strategies to treat women with poor ovarian response and primary ovarian insufficiency. *Curr Opin Obstet Gynecol*. 2021;33(3):241–8.
- Liu K, Rajareddy S, Liu L, et al. Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: New roles for an old timer. *Dev Biol*. 2006;299:1–11.
- Adhikari D, Liu K. Molecular mechanisms underlying the activation of mammalian primordial follicles. *Endocr Rev*. 2009;30:438–64.
- Kawamura K, Kawamura N, Hsueh AJ. Activation of dormant follicles: A new treatment for premature ovarian failure? *Curr Opin Obstet Gynecol*. 2016;28:217–22.
- Reddy P, Zheng W, Liu K. Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends Endocrinol Metab*. 2010;21:96–103.
- Hsueh AJ, Kawamura K, Cheng Y, Fauser BC. Intraovarian control of early folliculogenesis. *Endocr Rev*. 2015;36:1–24.
- Kawamura K, Cheng Y, Suzuki N, Takae S, et al. Hippo signaling disruption and AKT stimulation of ovarian follicles for infertility treatment. *Proc Natl Acad Sci USA*. 2013;110:17474–9.
- Cat Tuyen Vo K, Kawamura K. In vitro activation early follicles: From the basics sciences to the clinical perspectives. *Int J Mol Sci*. 2021;22(3785):1–18.
- Pan D. Hippo signaling in organ size control. *Genes Dev*. 2007;21:886–97.
- Zhao B, Tumaneng K, Guan KL. The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nat Cell Biol*. 2011;13:877–83.
- Meng Z, Moroishi T, Guan KL. Mechanisms of Hippo pathway regulation. *Genes Dev*. 2016;30:1–17.
- Grosbois J, Demeebare I. Dynamics of PI3K and Hippo signaling pathways during in vitro human follicle activation. *Hum Reprod*. 2018;33:1705–14.
- Wada K, Itoga K, Okano T, et al. Hippo pathway regulation by cell morphology and stress fibers. *Development*. 2011;138:3907–3914.
- Sansores-Garcia L, Bossuyt W, Wada K. Modulating f-actin organization induces organ growth by affecting the Hippo pathway. *EMBO J*. 2011;30:2325–35.
- Kawashima I, Kawamura K. Regulation of follicle growth through hormonal factors and mechanical cues mediated by hippo signaling pathway. *Syst Biol Reprod Med*. 2017;1–9.
- Cheng Y, Feng Y, Jansson L, et al. Actin polymerization-enhancing drugs promote ovarian follicle growth mediated by the hippo signaling effector YAP. *FASEB J*. 2015;29:2423–30.
- Shah JS, Sabouni R, Cayton Vaught KC, et al. Biomechanics and mechanical signaling in the ovary: A systematic review. *J Assist Reprod Genet*. 2018;35:1135–48.
- Grosbois J, Devos M, Demeebare I. Implications of nonphysiological ovarian primordial follicle activation for fertility preservation. *Endocrine Rev*. 2020;41(6):1–26.
- Suzuki N, Yoshioka N, Takae S, et al. Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency. *Hum Reprod*. 2015; 30(3):608–15.

27. Zhai J, Yao G, Dong F, et al. In vitro activation of follicles and fresh tissue auto-transplantation in primary ovarian insufficiency patients. *J Clin Endocrinol Metab.* 2016;101(11):4405–12.
28. Fabregues F, Ferreri J, Calafell JM, et al. Pregnancy after drug-free in vitro activation of follicles and fresh tissue autotransplantation in primary ovarian insufficiency patient: A case report and literature review. *J Ovarian Res.* 2018;11(1):76.
29. Ferreri J, Fabregues F, Calafell JM, Solernou R, Borras A, Saco A, et al. Drugfree in-vitro activation of follicles and fresh tissue auto-transplantation as a therapeutic option in patients with primary ovarian insufficiency. *Reprod Biomed Online.* 2020;40:254–60.
30. Kawamura K, Ishizuka B, Hsueh AJW. Drug-free in-vitro activation of follicles for infertility treatment in poor ovarian response patients with decreased ovarian reserve. *Reprod BioMed Online.* 2020;40:245–53.
31. Mahajan N, Kaur J, Bhattacharya B, et al. In vitro activation of ovary. *Onco Fertil J.* 2019;2:35.
32. Zhang X, Han T, Yan L, et al. Resumption of ovarian function after ovarian Biopsy/Scratch in patients with premature ovarian insufficiency. *Reprod Sci.* 2019; 26(2):207–13
33. Lundin SA, Pors SE, Kristensen SG, et al. Biopsying, fragmentation and autotransplantation of fresh ovarian cortical tissue in infertile women with diminished ovarian reserve. *Hum Reprod.* 2019; 34(10):1924–36.
34. Patel NH, Bhadarka HK, Patel NH, et al. Drug-free in vitro activation for primary ovarian insufficiency. *J Hum Reprod Sci* 2021;14(4):443–5.
35. Hsueh AJW, Kawamura K. Hippo signaling disruption and ovarian follicle activation in infertile patients. *Fertil Steril.* 2020;114(3):458–64.
36. Fabregues F, Ferreri J, Méndez M, et al. In vitro follicular activation and stem cell therapy as a novel treatment strategies in diminished ovarian reserve and primary ovarian insufficiency. *Front Endocrinol (Lausanne).* 2021;24(11):617704.
37. Wang W, Todorov P, Isachenko E, Rahimi G, Mallmann P, Wang M, Isachenko V. In vitro activation of cryopreserved ovarian tissue: A single-arm meta-analysis and systematic review. *Eur J Obstet Gynecol Reprod Biol.* 2021;258:258–64.
38. Ingber DE. Tensegrity: The architectural basis of cellular mechanotransduction. *Annu Rev Physiol.* 1997;59:575–99.
39. Matsuzaki S. Mechanobiology of the female reproductive system. *Reprod Med Biol.* 2021;20:371–401.
40. Thorne JT, Segal TR, Chang S, et al. Dynamic reciprocity between cells and their microenvironment in reproduction. *Biol Reprod.* 2015;92:25.
41. Atkinson LL, Martin F, Sturmey RG. Intraovarian injection of platelet-rich plasma in assisted reproduction: Too much Too soon? *Hum Reprod.* 2021;36(7):1737–50.
42. Sharara FI, Lelea LL, Rahman S, et al. A narrative review pf platelet-rich plasma (PRP) in reproductive medicine. *J Assist Reprod Genet.* 2021;38:1003–12.
43. Rosario R, Anderson RA. Novel approaches to fertility restoration in women with premature ovarian insufficiency. *Climacteric.* 2021;24(5):491–7.
44. Mawet M, Perrier S, Henry L, et al. Restoration of fertility in patients with spontaneous premature ovarian insufficiency: New techniques under the microscope. *J Clin Med* 2021;10(5647):1–9.
45. Demiray S, Yilmaz O, Goker ET, et al. Expression of the bone morphogenetic protein-2 (BMP2) in the human cumulus cells as a biomarker of oocytes and embryo quality. *J Hum Reprod Sci.* 2017;10:194.
46. Duncan WC, Van Den D S, Fraser HM. Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1a in the follicle and corpus luteum. *Endocrinology.* 2008;149:3313–20.
47. Ben-Haroush A, Abir R, Ao A, et al. Expression of basic fibroblast growth factor and its receptors in human ovarian follicles from adults and fetuses. *Fertil Steril.* 2005;84:1257–68.
48. Sfakianoudis K, Simopoulou M, Grigoriadis S, et al. Reactivating ovarian function through autologous platelet-rich plasma intra-ovarian infusion: Pilot data on premature ovarian insufficiency, perimenopausal, menopausal, and poor responder women. *JCM.* 2020b;9:1809.
49. Sills ES, Rickers NS, Li X, Palermo GD. First data on in vitro fertilization and blastocyst formation after intraovarian injection of calcium gluconate-activated autologous platelet rich plasma. *Gynecol Endocrinol.* 2018;34:756–60.
50. Farimani M, Heshmati S, Poorolajal J, et al. A report on three live births in women with poor ovarian response following intra-ovarian injection of platelet-rich plasma (PRP). *Mol Biol Rep.* 2019;46:1611–16.
51. Pacu I, Zygoiopoulos N, Dimitriu M, et al. Use platelet-rich plasma in the treatment of infertility in poor responders in assisted reproduction procedures. *Exp Ther Med.* 2021;22:1412–17.
52. Melo P, Navarro C, Jones C, et al. The use of autologous platelet-rich plasma (PRP) versus no intervention in women with low ovarian reserve undergoing fertility treatment: A non-randomized interventional study. *J Assist Reprod Genet.* 2020;37: 855–63.
53. Sfakianoudis K, Simopoulou M, Nitros N, et al. Autologous platelet-rich plasma treatment enables pregnancy for a woman in premature menopause. *JCM.* 2018;8:1.
54. Pantos K, Simopoulou M, Pantou AS, et al. Case series on natural conceptions resulting in ongoing pregnancies in menopausal and prematurely menopausal women following platelet- rich plasma treatment. *Cell Transplant.* 2019;28:1333–40.
55. Cakiroglu Y, Saltik A, Yuceturk A, et al. Effects of intraovarian injection of autologous platelet rich plasma on ovarian reserve and IVF outcome parameters in women with primary ovarian insufficiency. *Aging (Albany NY).* 2020;12:10211–22.
56. Hsu CC, Hsu L, Hsu I, et al. Live birth in woman with premature ovarian insufficiency receiving ovarian administration of platelet-rich plasma (PRP) in combination with gonadotropin: A case report. *Front Endocrinol (Lausanne).* 2020;11:1–5.
57. Hosseini L, Shirazi A, Naderi MM, et al. Platelet-rich plasma promotes the development of isolated human primordial and primary follicles to the preantral stage. *Reprod Biomed Online.* 2017;35:343–50.
58. de Miguel-Gomez I, Lopez-Martinez S, Campo H, et al. Comparison of different sources of platelet-rich plasma as treatment option for infertility-causing endometrial pathologies. *Fertil Steril.* 2020;115:490–500.
59. Spanou S, Kalogiannis D, Zapanti E, et al. Interleukin 15 concentrations in follicular fluid and their effect on oocyte maturation in subfertile women undergoing intracytoplasmic sperm injection. *J Assist Reprod Genet.* 2018;35:1019–25.
60. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature.* 2004;428:145–50.
61. Zou K, Yuan Z, Yang Z, et al. Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nat Cell Biol.* 2009;11:631–6.
62. Yuan JH, Zhang DD, Wang L, et al. No evidence for neo-oogenesis May link to ovarian senescence in adult monkey. *Stem Cells.* 2013;31:2538–50.
63. Wagner M, Yoshihara M, Douagi L, et al. Single-cell analysis of human ovarian cortex indentifies distinct cell populations but no oogonial cells. *Nat Com.* 2020;11:1147–67.
64. Mendelson A, Frenette PS. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nat Med.* 2014;20(8): 833–46.
65. Jung Y, Brack AS. Cellular mechanisms of somatic stem cell aging. *Curr Top Dev Biol.* 2014;107:405–38.
66. Ye H, Zheng T, Li W, Li X, Fu X, Huang Y, et al. Ovarian stem cell nest in reproduction and ovarian aging. *Cell Physiol Biochem.* 2017;43:1917–25.

67. Polonio A, Garcia-Velasco JA, Herraiz S. Stem cell paracrine signaling for treatment of premature ovarian insufficiency. *Front Endocrinol.* 2021;11:626322.
68. Lee HJ, Selesniemi K, Niikura Y, et al. Bone marrow transplantation generates immature oocytes and rescues longterm fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. *J Clin Oncol.* 2007;25(22):3198–204.
69. Liu J, Zhang H, Zhang Y, et al. Homing and restorative effects of bone marrow-derived mesenchymal stem cells on cisplatin injured ovaries in rats. *Mol Cells.* 2014;37(12):865–72.
70. Herraiz S, Buigues A, Diaz-Garcia C, et al. Fertility rescue and ovarian follicle growth promotion by bone marrow stem cell infusion. *Fertil Steril.* 2018;109(5):908–18.
71. Ding L, Yan G, Wang B, et al. Transplantation of UC-MSCs on collagen scaffold activates in dormant ovaries of POF patients with long history of infertility. *Sci China Life Sci.* 2018;61:1554–65.
72. Yan L, Wu Y, Li L, et al. Clinical analysis of human umbilical cord mesenchymal stem cell allotransplantation in patients with premature ovarian insufficiency. *Cell Prolif.* 2020;53:e12938.
73. Edessy M, Hosni H, Shady Y. Autologous stem cells Therapy: The first baby of idiopathic premature ovarian failure. *Acta Med Int.* 2016;3:19–23.
74. Gabr H, Elkheir WA, El-Gazaar A. Autologous stem cell transplantation in patients with idiopathic premature ovarian failure. *J Tissue Sci Eng.* 2016;7(Suppl):3.
75. Gupta S, Lodha P, Karthick MS, Tandulwadkar SR. Role of autologous bone marrow-derived stem cell therapy for follicular recruitment in premature ovarian insufficiency: Review of literature and a case report of world's first baby with ovarian autologous stem cell therapy in a perimenopausal woman of age 45 year. *J Hum Reprod Sci.* 2018;11:125–30.
76. Pellicer N, Herraiz S, Romeu M, et al. Bone marrow derived stem cells restore ovarian function and fertility in premature ovarian insufficiency women. Interim report of a randomized trial: Mobilization versus ovarian injection. *Hum Reprod.* 2020;35:38–9.
77. Mashayekhy M, Mirzadeh E, Chekini Z, et al. Evaluation of safety, feasibility and efficacy of intraovarian transplantation of autologous adipose derived mesenchymal stromal cells in idiopathic premature ovarian failure patients: Non-randomized clinical trial, phase I, first in human. *J Ovarian Res.* 2021;14:5.
78. Chiang JL, Shukla P, Pagidas K, et al. Mitochondria in ovarian aging and reproductive longevity. *Ageing Res Rev.* 2020;63:101168.
79. Wang L-Y, Wang DH, Zou XY, et al. Mitochondrial functions on oocytes and preimplantation embryos. *J Zhejiang Univ Sci B.* 2009;10:483–92.
80. Lin DP-C, Huang C-C, Wu H-M, et al. Comparison of mitochondrial DNA contents in human embryos with good or poor morphology at the 8-cell stage. *Fertil Steril.* 2004;81:73–9.
81. Labarta E, de los Santos MJ, Escriba MJ, et al. Mitochondrial as a tool for oocyte rejuvenation. *Fertil Steril.* 2019;111:219–26.
82. Rodriguez Varela C, Herraiz S, Labarta E. Mitochondrial enrichment in infertile patients: A review of different mitochondrial replacement therapies. *Ther Adv Reprod Health.* 2021;15:1–16.
83. Seli E. Mitochondrial DNA as a biomarker for in-vitro fertilization outcome. *Curr Opin Obstet Gynecol.* 2016;28:158–63.
84. Cohen J, Scott R, Schimmel T, et al. Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs. *Lancet.* 1997;350(9072):186–7.
85. Cohen J, Scott R, Alikani M, et al. Ooplasmic transfer in mature human oocytes. *Mol Hum Reprod.* 1998;4:269–80.
86. Huang C-C, Cheng T-C, Chang H-H, et al. Birth after the injection of sperm and the cytoplasm of tripronucleate zygotes into metaphase II oocytes in patients with repeated implantation failure after assisted fertilization procedures. *Fertil Steril.* 1999;72:702–6.
87. Dale B, Wilding M, Botta G, et al. Pregnancy after cytoplasmic transfer in a couple suffering from idiopathic infertility. *Hum Reprod.* 2001;16:1469–72.
88. Tanaka A, Nagayoshi M, Awata S, et al. Metaphase II karyoplast transfer from human in-vitro matured oocytes to enucleated mature oocytes. *Reprod Biomed Online.* 2009;19:514–20.
89. Zhang J, Liu H, Luo S, et al. Live birth derived from oocyte spindle transfer to prevent mitochondrial disease. *Reprod Biomed Online.* 2017;34:361–8.
90. Zoon K. Letter to sponsors/researchers – Human Cells Used in Therapy Involving the Transfer of Genetic Material by Means Other than the Union of Gamete Nuclei. Rockville, MD: Food and Drug Administration, 2001.
91. Brenner CA, Barritt JA, Willadsen S, et al. Mitochondrial DNA heteroplasmy after human ooplasmic transplantation. *Fertil Steril.* 2000;74:573–8.
92. Cree L, Loi P. Mitochondrial replacement: From basic research to assisted reproductive technology portfolio tool-technicalities and possible risks. *Mol Hum Reprod.* 2015;21:3–10.
93. White YAR, Woods DC, Takai Y, et al. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat Med.* 2012;18:413–21.
94. Woods DC, Tilly JL. Autologous germline mitochondrial energy transfer (AUGMENT) in human assisted reproduction. *Semin Reprod Med.* 2015;33:410–21.
95. Fakih MH. The AUGMENT treatment: Physician reported outcomes of the initial global patient experience. *J Fertil Vitr.* 2015;3:3.
96. Oktay K, Baltaci V, Sonmezler M, et al. Oogonial precursor cell-derived autologous mitochondria injection to improve outcomes in women with multiple IVF failures due to low oocyte quality: A clinical translation. *Reprod Sci.* 2015;22:1612–7.
97. Labarta E, de Los Santos MJ, Herraiz S, et al. Autologous mitochondrial transfer as a complementary technique to intracytoplasmic sperm injection to improve embryo quality in patients undergoing in vitro fertilization: A randomized pilot study. *Fertil Steril.* 2019;111:86–96.

REPEATED IMPLANTATION FAILURE

David Reichman, Hey-Joo Kang, and Zev Rosenwaks

Overview

Human reproduction is inherently inefficient, as evidenced by a mean delivery rate of 45.4% per initiated *in vitro* fertilization (IVF) cycle with embryo transfer in the United States in 2019 [1]. Despite advances in assisted reproductive technologies over the past four decades, patients remain who fail to achieve live births following multiple IVF/embryo transfer cycles. Although delivery rates have continued to improve, no identifiable aetiology is found in the majority of failed implantation cycles. Thus, isolating a specific cause for repeated implantation failure (RIF) can be challenging. Patient age, euploidy of embryos, culture conditions, and endometrial receptivity have all been implicated in RIF.

Maternal age remains the single most important variable in predicting successful implantation. Human females have six to seven million oocytes in utero at 20 weeks of gestation, and through atresia, this is reduced to one to two million at birth and 400,000 at menarche. Meiotic errors in chromosome segregation increase with female age, in turn, increasing the aneuploidy rate of resultant embryos. Advanced female age is not only associated with an increase in embryo aneuploidy and a decline in ovarian reserve, it also results in a diminished response to gonadotropin stimulation. Ovarian reserve testing can help anticipate response to gonadotropin stimulation and may correlate with success rates; however, it falls short of predicting cycle outcome. This has been shown in both fertile and infertile couples, as well as in single women using donor sperm [2]. A wide range of individuals with normal to diminished ovarian reserve appear to achieve comparable pregnancy rates within three months of treatment.

Embryos have a high attrition rate in the laboratory and following implantation, mostly due to genetic abnormalities. *In vitro*, this rate can be amplified by culture conditions as well as perturbations associated with handling and exposure to ambient air. The introduction of closed incubator systems utilizing continuous time-lapse monitoring of embryos in stable non-disturbed culture conditions has mitigated the impact of the artificial laboratory environment on embryos. Despite these advances in laboratory conditions, it is reasonable to assume that some degree of embryonic loss may be due to the artificial environment. Once embryos have been selected based on developmental competence, embryonic loss still continues after transfer. Even euploid embryos in older women have a decreased clinical pregnancy rate and live birth rate for reasons owing to lower embryo quality and stamina in the lab [3].

There is no universally accepted definition of RIF, and the definition has continued to evolve with the advent of new approaches to ART [4]. RIF was historically defined by the absence of implantation after three or more transfers of high-quality embryos, or after transfer of >10 high quality embryos in multiple cycles. When specifically referring to PGT-A cycles, recurrent

implantation failure can be defined as a lack of sustained implantation after transfer of more than three high morphologic quality euploid blastocysts [5, 6]. Although these definitions may be somewhat arbitrary, they can serve as guides to demarcate this group of challenging patients. This chapter summarizes specific causes of RIF as well as treatment strategies designed to improve the efficiency of embryo implantation.

Parental genetics

Translocations represent a variety of rearrangements between non-homologous chromosomes. They can be reciprocal, whereby two non-homologous chromosomes exchange segments, or Robertsonian, when two acrocentric chromosomes break at their centromere to fuse as a single, large chromosome with the loss of the two short arms. Parental translocations often affect the pattern of segregation during meiosis, resulting in a variety of aneuploidies, depending upon which chromosomes are involved as well as the size of the rearrangement.

Although only a small portion of couples with RIF will have abnormal karyotypes, the incidence of parental chromosomal abnormalities is reported to be 2.5% [7]. By comparison, the incidence of translocation carriers in couples with recurrent pregnancy loss was found to be 4.7%. In a study examining extreme cases of RIF (either six or more failed IVF attempts or 15 or more transferred embryos), 10 of 65 couples (15.4%) were noted to have either chromosomal translocations, mosaicism, inversions, or deletions [8]. Another study of 317 couples confirmed a 2.1% incidence of karyotype abnormalities, consistent with prior findings [9].

In light of these observations, parental karyotypes should be considered in couples with RIF. Couples with known translocations should be carefully counselled with the aim of providing advice regarding PGT-A.

Blastocyst culture

Identification of embryos with a higher implantation potential is key to improving the efficiency of IVF. It is widely acknowledged that cleavage-stage embryo development is controlled by maternal RNA transcripts until the 4–8-cell stage [10]. Activation of the embryonic genome begins on day 3 of development and continues to the blastocyst stage, a stage that confers a higher implantation potential than cleavage-stage embryos. Early attempts to culture embryos to the blastocyst stage used monophasic cultures with unsatisfactory blastulation rates. Sequential culture systems evolved in the 1990s with an increased understanding of physiologic conditions *in vivo*. The first culture stage (pronuclear stage to compaction) consists of non-essential amino acids, EDTA, and pyruvate and a reduced glucose concentration. The second culture stage (compaction to blastocyst) adds essential amino acids, removes EDTA, reduces the pyruvate concentration, and

increases glucose concentration to meet the increased energy demands of the embryo during rapid cell division. Sequential culture media thus facilitates the selection of embryos most suitable for transfer. Global culture media have been developed, which are also suitable for efficient blastocyst development.

Although a high proportion of embryos fail to form blastocysts due to genetic aneuploidy, a subset of embryos arrest at the cleavage stage due to suboptimal culture conditions. Earlier studies suggesting a higher implantation rate with blastocyst transfers may have used select patient populations with favourable prognoses for implantation. The ideal candidates for blastocyst transfer are high ovarian responders to gonadotropins who create excess embryos, allowing one to select the best available blastocysts to enhance implantation rates. Conversely, marginal or poor responders with a limited number of embryos are not good candidates for prolonged culture conditions, as they may arrest at cleavage stages prior to transfer. Many patients with RIF fall into the latter category, where prolonged culture of a small number of embryos appears to offer no significant advantages. For those who are high responders, prolonged culture conditions may improve the implantation rate and clinical success, and opens the possibility of performing pre-implantation genetic screening when indicated.

Embryo genetics

A direct correlation between female age and oocyte aneuploidy exists, with the steepest rise in aneuploidy occurring in the late thirties and early to mid-forties. This association is derived from cytogenetic analysis of products of conception from first trimester miscarriages, as well as aneuploidy assessment of biopsied embryos [11]. In original studies using fluorescent *in situ* hybridization (FISH) to diagnose numeric abnormalities of X, Y, 18, 13, and 21, Munne noted aneuploidy rates for these five chromosomes to be 37% for women aged 40–47. With the advent of comparative genomic sequencing (CGH) for all 24 chromosomes, greater aneuploidy rates were appreciated, ranging from 58% at age 40 to 100% at age 47 [12]. Testing has evolved to next generation sequencing (NGS), which is the current gold standard for PGT-A. Aneuploidy rates as high as 49.7% for women aged 35–37, 61.7% for women aged 38–40, and >75% for women aged >43 years are observed with such testing [13]. These numbers give a false reassurance of euploid rates/embryo in older women, as many women approaching their mid-forties do not make a sufficient number of blastocysts to be included in these statistics. This testing underscores the importance of female age in predicting the implantation potential of embryos.

As embryo culture techniques and the efficiency of reaching the blastocyst stage *in vitro* have improved, pre-implantation genetic testing by trophectoderm biopsy has become, for better or worse, the prevailing approach to care for many patients. PGT-A itself has been useful as a technique to demonstrate the true incidence of recurrent implantation failure, which may be lower than originally thought. In a study of 4429 patients (average age 35.4) undergoing frozen euploid single embryo transfers, the sustained implantation rate after three consecutive transfers was 95.2%, suggesting that true recurrent implantation failure in patients who are good candidates for PGT-A is relatively rare [5].

It remains unclear whether patients with RIF exhibit a higher proportion of aneuploid embryos, or indeed whether these patients benefit from PGT-A at all. The earliest studies examining the role of PGT-A in patients with RIF employed the

use of FISH; however, by virtue of being unable to identify all chromosomes, this method was prone to limited interpretation [14, 15]. More recent studies examining the role of PGT-A in patients specifically with RIF have used either array CGH or NGS for trophectoderm analysis. Greco et al. failed to demonstrate a difference in the incidence of aneuploidy in 43 RIF compared to 45 infertile good-prognosis patients (aneuploidy 53.8% vs 48.2%, respectively) with the use of PGT-A testing via array CGH [16]. Although the incidence of aneuploidy was not significantly different, the clinical pregnancy rate increased to 68.3% with testing versus 21.2% for RIF patients not undergoing testing. In a multicentre prospective pilot study from 2018 involving 92 patients with RIF, there were no differences in the live birth rates per started cycle with the use of PGT-A, although the live birth rate per embryo transfer was higher with PGT-A testing than without (62.5% vs 31.7%, respectively) [17]. Conversely, some studies have suggested that there is a negative impact of PGT-A in RIF patients, as they exhibited lower live birth rates after biopsy when compared to controls, potentially as a result of biopsy-induced embryo damage [18].

Thus, the exact role for PGT-A in patients with RIF remains to be determined. The potential inaccuracy of FISH-based technology and the developmental susceptibility of day 3 embryos to injury incurred by biopsy might have explained the earliest studies' failures to demonstrate benefit for IVF patients. Trophectoderm biopsy has several advantages over blastomere biopsy, including greater developmental resiliency, less mosaicism, and the ability to analyse multiple cells. Moreover, the greater accuracy of 24 chromosome analysis has been well validated [19]. Blastocyst-based PGT-A has been suggested to improve the efficiency of IVF in older individuals (>40) [20]. Another retrospective study claimed a benefit in implantation and live births when PGS was used in women aged 40–43 with multiple prior IVF failures, with a live birth rate for PGS-FET (45.5%) significantly greater than for fresh transfer without PGS (15.8%) or frozen transfer of non-PGS embryos (19.0%) [21]. More recent data, however, has again called into question the utility of universal PGT-A for the good-prognosis patient: the STAR study group demonstrated via prospective RCT that for women <40 years old, PGT-A did not improve ongoing pregnancy rate per initiated cycle [22]. This was followed by a multicentre randomized trial of sub-fertile women 20–37 years of age with three or more good quality blastocyst, with the study finding no benefit in cumulative live birth rate using PGT-A [23]. In this study, miscarriage rates were also similar between the groups: 8.7% in the PGT-A versus 12.6% in the non-PGT-A group. The benefit of PGT-A for young patients, and specifically those young patients with RIF, is less established and will need further study.

It may be beneficial to perform a blastocyst biopsy with 24 chromosome PGT-A to reduce the incidence of viable trisomies and spontaneous pregnancy loss, both of which affect older patients (>38 years old) disproportionately. PGT-A should be considered for patients experiencing repeated implantation failure, as it may provide important information regarding the incidence of aneuploidy in these susceptible individuals. Biopsy and analysis do not, however, intrinsically increase the implantation potential of any given euploid embryo, and indeed add cost, invasiveness, and the potential for discarding a normal embryo; thus, PGT-A should not be viewed as a reflex intervention for RIF patients, but carefully selected candidates may benefit from this approach to isolate an embryo versus uterine cause for implantation failure.

Sperm genetics

Sperm concentration, motility, and morphological assessment are relatively poor predictors of conception with assisted reproductive technology. Recently, tests of sperm DNA integrity have been increasingly used for evaluating spermatozoa in conjunction with semen analyses. Several studies have provided evidence that sperm DNA damage is correlated with poor reproductive outcome, including increased pregnancy loss and chromosomal aneuploidy [24]. Damage of sperm DNA has also been associated with poor development of embryos, and both animal and human studies have implied that failure to achieve conception may be associated with markedly elevated sperm DNA fragmentation [25–29]. While elevated DNA fragmentation has been associated with an increase in miscarriage risk in spontaneous pregnancies, its role in patients with RIF remains uncertain [30, 31].

Fragmentation is a break in the DNA strands and can be associated with obesity, smoking, urogenital infections, advanced age, varicoceles, and any number of environmental and medical factors. There are several assays to measure sperm DNA and chromatin damage. The most common are: the sperm chromatin structure assay (SCSA), single cell gel electrophoresis (COMET), terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labeling (TUNEL), and sperm chromatin dispersion (SCD) assay. Some assays have undergone more rigorous testing than others, and no assay is able to differentiate clinically important DNA damage from insignificant damage.

Sperm DNA damage is lower in the seminiferous tubules as compared to epididymal or ejaculated spermatozoa [32–34]. In RIF couples in whom high DNA fragmentation has been documented, the use of testicular retrieved spermatozoa has been suggested by some researchers [32, 35]. Greco et al. studied 18 couples with at least two unsuccessful IVF/ICSI attempts where male partners had ejaculated spermatozoa with >15% DNA damage by TUNEL assay. The incidence of DNA fragmentation in their testicular sperm (4.8%) was markedly lower as compared to ejaculated specimens from the same individuals (23.6%), with eight subsequent clinical pregnancies (44.4% clinical pregnancy rate) when testicular sperm was used for ICSI. More recent studies, however, failed to show an association between elevated DFI and RIF [36]. The largest study to date examined 1216 cycles and showed no association between DNA fragmentation and the incidence of RIF [37].

A number of techniques have been proposed to select spermatozoa with lower levels of DNA damage from ejaculated samples, including annexin-V columns, sperm hyaluronic acid binding, confocal light absorption scattering spectroscopy, and high-magnification ICSI (IMSI) [38–41]. IMSI has been proposed as a useful intervention for patients with repeated implantation failure [41–44]. Spermatozoa noted to have large vacuoles under high-power magnification have increased DNA defects [45–48]. The use of IMSI to select spermatozoa that are free of vacuoles has been suggested to improve embryo quality at early cleavage stages and to potentially increase implantation and pregnancy [41, 43]. Subsequent studies, however, have failed to demonstrate different ongoing pregnancy, miscarriage, or live birth rates with IMSI when compared to conventional ICSI [49]. A study specifically examining the use of IMSI (8400 \times magnification to select morphologically normal sperm) in 200 patients with RIF failed to demonstrate any significant benefits in terms of fertilization, implantation, or pregnancy rates when IMSI was employed as compared to conventional ICSI [44]. Given the mixed nature

of existing data, further studies are required to assess whether sperm DNA fragmentation testing is warranted in patients experiencing RIF. A method of sperm selection called ZyMot uses a microporous membrane to select for sperm with minimal DNA fragmentation to identify sperm for use in ICSI. Some clinics have adopted this for use in all ICSI cases to minimize the need for sperm DNA integrity testing altogether. Studies are needed to further assess the optimal methods of testing, and whether testicular sperm extraction, IMSI, ZyMot, or similar techniques offer any significant benefit over use of ICSI with ejaculated specimens. Currently, there is insufficient data to routinely suggest such interventions.

Along with sperm DNA fragmentation, sperm aneuploidy may also play a role in recurrent implantation failure. Men with abnormal semen parameters have been noted to have increased aneuploidy rates in randomly collected semen samples. Burrello sampled 48 consecutive male patients to evaluate aneuploidy rates in swim-up preparation used for ICSI. Sperm was evaluated with 5-probe FISH and divided into two groups: those with sperm aneuploidy rates in the normal range versus a group with aneuploidy rates above the upper limit of normal (as determined by WHO criteria). Men with lower sperm aneuploidy demonstrated higher implantation (34% vs 13%) and pregnancy (75% vs 34%) rates as well as lower miscarriage rates (38% vs 11%) [50]. Retrospective studies have suggested that couples with higher rates of sperm aneuploidy may have higher rates of embryo aneuploidy and may benefit from the use of PGT-A [51]. Such data has not been substantiated prospectively. Given that, at this point, individually tested sperm is unable to be used for fertilization, the routine clinical utility of sperm aneuploidy testing remains to be established.

Uterine pathology

An evaluation of the uterine cavity is warranted in patients who have experienced repeated IVF failures after transfer of high-quality embryos. Fibroids, polyps, intrauterine adhesions, chronic endometritis, or Mullerian anomalies have all been implicated in RIF. The incidence of previously unrecognized intrauterine pathology in individuals with RIF may be elevated; in fact, in some studies it was as high as 25%–50% [52].

There are a number of theories regarding the mechanisms by which fibroids adversely affect implantation: mechanical obstruction of tubal ostia, chronic intracavitary inflammation, and increased uterine contractility are the most commonly cited [53–55]. It is generally accepted that subserosal myomas do not adversely affect pregnancy or live birth rates, and thus removal is not warranted to improve fertility. It is equally agreed that submucous myomas decrease pregnancy rates and increase the incidence of miscarriage. For the majority of patients with submucous myomas, surgical resection restores pregnancy rates to match those of infertile women without myomas. While the benefits of submucous myoma resection are clear, the benefit of myomectomy for non-distorting intramural myomas greater than 2 cm located outside of the endometrial cavity is more controversial [56, 57]. The largest published meta-analysis suggests a 21% relative reduction in live birth rate in women with non-cavity distorting intramural myomas as compared to women without myomas, but there is no clear evidence that removal of these fibroids leads to higher pregnancy rates [58]. There is also emerging evidence that the endometrium overlying intramural myomas has altered receptivity and gene expression of TGF- β 3 and HOXA-10. Larger

fibroids produce greater quantities of TGF- β 3, allowing more to be released by the overlying endometrial cells. This in turn can alter BMP-2 and HOXA-10 expression and reduce the implantation rate of a euploid embryo [59].

Endometrial polyps may be a cause of reduced implantation rates [60]. Multiple retrospective studies have reported improved spontaneous conception rates when endometrial polyps are resected [61–63]. A randomized controlled trial of 215 infertile women with polyps, which compared a group undergoing polypectomy with those undergoing a diagnostic hysteroscopy without intervention revealed that pregnancy was 2.1 times more likely (95% CI 1.5–2.9) after polyp resection [64]. Previous uterine instrumentation, especially those complicated with pelvic infection, should prompt investigation for intrauterine adhesions. In one study of patients with RIF undergoing hysteroscopy, the incidence of intrauterine adhesions was 8.5%. Available evidence suggests that subsequent surgical correction improves fertility outcomes [65–69].

Chronic endometritis should be excluded in patients with repeated implantation failure without apparent cause. This inflammatory condition of the endometrium is associated with reduced uterine receptivity via dysregulated lymphocyte activity and abnormal expression of cytokines and other regulatory molecules [70, 71]. Unfortunately, there remains no consensus on a formal definition of chronic endometritis, nor is there a defined threshold of CD138+ plasma cells on histologic analysis that satisfies the diagnosis. Nevertheless, several researchers have implicated endometrial inflammation as a potential aetiology of RIF [72–74]. The incidence of chronic endometritis based on histological evidence of plasma cells has been estimated to be as high as 30.3% in patients with RIF.

Indeed, lower implantation rates have been noted in patients with evidence of endometritis [75]. One well-designed study compared IVF outcomes in RIF patients after successful treatment of endometritis versus RIF patients in whom evidence of endometritis persisted despite three rounds of antibiotic treatment [75]. Biopsies were performed in the follicular phase using a 3-mm curette attached to a 20-mL syringe, with samples divided into equal aliquots for culture and histologic analysis. Patients cultured positive for Gram negative bacteria were treated with ciprofloxacin for 10 days, whereas those with Gram positive bacteria were treated with an 8 day course of amoxicillin and clavulanate. Patients with histologic evidence of endometritis in the absence of positive cultures were treated with a single dose of intramuscular ceftriaxone followed by a 14-day course of oral doxycycline and metronidazole. Live birth rates were 60.8% in the patients in whom endometritis was successfully treated (based on negative repeat culture and histology), versus 13.3% for those in whom evidence of endometritis persisted after treatment. A meta-analysis has suggested that treatment of chronic endometritis in individuals with RIF may improve outcomes, but such analyses are limited by the observational nature of the majority of studies and the small number of studies contributing to the analysis [76]. In the most recent study, 640 IVF patients underwent endometrial biopsy for CD138 analysis: >5 CD138+ cells was associated with lower implantation rates (32.3% vs 51.6%) and live birth rates (30.7% vs 52.1%), with a cure rate of 89% for patients undergoing antibiotic treatment [77]. Based on the prevailing data, there exists enough evidence to either perform an endometrial biopsy to rule out chronic endometritis or empirically treat for presumed endometritis without an endometrial biopsy (an approach that can be seen as controversial).

Another condition to consider as a potential cause for repeated implantation failure is adenomyosis, defined by endometrial gland invasion of the uterine myometrium [78, 79]. While ultrasound findings can be suggestive of adenomyosis, the most definitive diagnoses can be made with an MRI with contrast. Rates of implantation, clinical pregnancy per cycle, and live birth have all been demonstrated to be reduced in patients suffering from either focal or diffuse adenomyosis [80, 81]. Though adenomyosis has been implicated as having a significant negative affect on female fertility and obstetrical outcomes, the condition is one of the least treatable of all uterine pathologies [82–84]. Limited success has been reported in women with adenomyosis-associated RIF in studies utilizing ultra-long pituitary GnRH agonist down-regulation prior to IVF. Nonetheless, the data is limited, and further corroboration is needed [78, 84].

Given the extent to which uterine pathology can be implicated in repeated implantation failure, diagnostic investigation of the myometrium and endometrial cavity is warranted in all patients with RIF. Hysterosalpingogram, saline infusion sonography, 3D ultrasonography, MRI, and hysteroscopy are all suitable for evaluation of uterine architecture and the endometrial cavity. The incidence of abnormal findings encountered at time of hysteroscopy in patients with RIF has ranged between 14% and 51% [85–90]. Some have argued that saline hysterography (SHG) offers similar detection rates with less invasiveness and cost; in one study, SHG detected all but one uterine abnormality, missing only a small endometrial polyp [91]. Similarly, hysterosalpingography provides data both on the endometrial cavity as well as information on the status of the fallopian tubes (i.e. hydrosalpinges); however, even these modalities may miss small intrauterine lesions [92]. A prospective study comparing vaginal sonogram, SHG, and diagnostic hysteroscopy concluded that hysteroscopy offered a more thorough method for detecting intracavitary lesions than SHG or transvaginal ultrasound [93]. A subsequent prospective multicentre RCT, however, compared 350 RIF patients undergoing hysteroscopy to 352 RIF controls who did not undergo hysteroscopy; only a 4% incidence of surgically correctable pathology was found, with no difference in live birth rates between the groups after surgical correction [94].

Molecular or transcriptomic testing of the endometrium is an emerging diagnostic strategy for women suffering from RIF. Research has suggested that patients with RIF exhibit an increase in pro-inflammatory markers such as resistin, leptin, and IL-22 on mid-secretory endometrial biopsies, as well as altered T-lymphocytes [95]. Endometrial prostaglandin synthesis has also been proposed to be aberrant in women with RIF [96]. The transcriptomic profile of the endometrium throughout the menstrual cycle has allowed for the potential molecular identification of receptive endometria, and has allowed for analysis of dysregulated genes in women exhibiting RIF [97–103].

Transcriptomic studies suggest the endometrial expression profile in patients with RIF is altered as compared to fertile control subjects [104]. Diaz-Gimeno et al. (the IVI Group) developed an endometrial receptivity array (ERA) examining 238 endometrial genes, which is reportedly capable of identifying a receptive endometrium in both natural and stimulated cycles [105]. The ERA requires an endometrial biopsy to be performed at the time of a potential transfer, either in a natural cycle or an oestrogen-progesterone programmed cycle. The sample is either “receptive,” indicating that transfer should occur in a subsequent cycle at the same time as the initial biopsy, or “non-receptive,” with either a pre- or post-receptive interpretation. Non-receptive samples

receive a recommendation for an altered length of progesterone exposure to meet the patient's individualized window of implantation. This array has been reported to be more accurate than traditional histology and appears to be reproducible within the same patient up to 40 months after first analysis [106]. Based on the results of these analyses, the window of implantation is variable and unique to the individual patient; the conclusion is that a "one-size-fits all" approach to the timing of embryo transfer may not benefit the patient, whose transcriptomic endometrial profile is altered [107].

Small retrospective studies have suggested that patients with a previously failed euploid FET have a high incidence (22.5%) of window of implantation displacement, with reported improvements in implantation and ongoing pregnancy rates when "personalized" embryo transfers are performed [108]. In a preliminary study, 85 RIF patients and 25 controls underwent endometrial sampling and transfer guided by ERA result. 74.1% of patients in the RIF group had a "receptive" result on initial ERA, as compared to 88% of control subjects. In 15 of 22 RIF patients with "non-receptive" ERA results, a second ERA demonstrated a displaced implantation window; 8 of these 15 patients subsequently conceived following embryo transfer timed according to the window of implantation identified by the second ERA [109].

Data has been mixed regarding the clinical utility of personalized embryo transfers via the ERA test. A Japanese retrospective study of 50 patients with RIF undergoing ERA suggested a benefit to personalized timing for embryo transfer in 12 patients who were found to be non-receptive on initial ERA [110]. The IVI group, presenting data on a five-year RCT examining "personalized" ERA-guided embryo transfer, concluded that patients undergoing personalized transfer exhibited improved clinical outcomes [111]. However, there was no difference in outcomes when an intention to treat analysis was performed; thus, the methodology of this study has been called into question [112]. Along such lines, a retrospective study of 253 patients with RIF failed to demonstrate an improvement in outcomes with the use of the ERA test [113]. In a prospective study of 228 single euploid FETs, "personalized" transfer using ERA in 87 patients in whom initial biopsy was non-receptive failed to produce higher live birth rates when compared to patients undergoing FET with standard synchronization protocols (56.5% vs 56.5%, respectively) [114].

Some practitioners have begun to perform the ERA test before patients have undergone their first transfer, even though convincing data justifying such an approach is lacking [115]. Before the ERA is routinely applied to daily practice, further, large confirmatory studies are required to substantiate its usefulness. Until then, the routine use of the endometrial receptivity array as a first-line diagnostic test for patients who have never undergone transfer should not be endorsed. For patients with both infertility and recurrent implantation failure, follow-up studies beyond the initial sponsored study have failed to show that ERA biopsies improve implantation [113, 114]; however, for patients who have failed two transfers of euploid embryos, counselling the patient about the availability of ERA is reasonable.

Tubal pathology

In individuals with repeated implantation failure, tubal pathology must also be excluded. The mechanisms whereby hydrosalpinges adversely affect reproduction are potentially multifactorial: accumulated tubal fluid may exert a direct embryotoxic effect, may act to mechanically flush an embryo from the uterus, or

adversely alter endometrial receptivity [116]. Evidence suggests that live-birth rates in patients with hydrosalpinges undergoing IVF are reduced [117–119]. A direct effect on the endometrium was suggested by a study by Seli et al., in which deranged expression of leukaemia inhibitory factor, an endometrial cytokine, was restored to normal following salpingectomy [120]. Avb3 integrin expression is similarly restored following salpingectomy [121]. A multicentre prospective randomized trial revealed pregnancy rates of 23.9% and live-birth rates of 16.3% in IVF patients in whom hydrosalpinges were left untreated as compared to 36.6% and 28.6%, respectively, when salpingectomy was performed prior to IVF [118]. The greatest effect was noted in women in whom hydrosalpinges were evident on transvaginal ultrasound or when a tubal diameter of greater than 3 cm was visualized under fluoroscopy. In women with distorted anatomy due to endometriosis or prior PID, IVF outcomes in women with proximal tubal occlusion are comparable to those following salpingectomy [122]. Given the negative impact of tubal pathology, it is advisable to exclude hydrosalpinges in women with RIF, regardless of the initial infertility diagnosis.

Thrombophilia

Although some clinicians will include a thrombophilia panel in patients with RIF, the relationship between coagulation abnormalities and RIF is far from established. Such testing has been largely influenced by limited data on patients with recurrent pregnancy loss rather than RIF. While several studies have examined a potential role of inherited or acquired thrombophilia in patients with RIF, their clinical relevance and accuracy have been questionable [123–125]. The largest study to date examined 594 women with RIF who underwent thrombophilia testing as compared to 637 fertile patients and showed no association between the common thrombophilias (activated protein C resistance, Factor V Leiden, prothrombin mutation, APL antibodies) and RIF [126]. Even when antiphospholipid or antinuclear antibody positivity is present in patients with RIF, the benefit of anticoagulants remains unclear. Prospective data has been mixed, with one study employing heparin and aspirin showing no benefit, and another using low molecular weight heparin (LMWH) demonstrating higher implantation and live birth rates [127, 128]. LMWH has also been evaluated in RIF patients with negative thrombophilia serology, with one small RCT suggesting a trend towards benefit for patients undergoing prophylactic anticoagulation [129]. Currently, there is insufficient data to recommend the routine use of aspirin or LMWH for patients with RIF; however, large, randomized controlled studies are warranted to further evaluate the possible benefits in the appropriate research setting.

Techniques

Assisted hatching

Embryos subjected to *in vitro* culture conditions may undergo physicochemical changes of the zona pellucida, including zona hardening, which may hinder zona hatching, blastocyst expansion, and implantation [130, 131]. Cleaved embryos with reduced zona thickness have higher implantation rates than those with thick zonae. Thus, it was suggested that either artificially opening or thinning the zona could facilitate the hatching process [132–134]. A variety of techniques have subsequently been developed to aid in the hatching process, including mechanical partial zona dissection, chemical drilling using acid Tyrode solution, enzymatic thinning, laser-assisted hatching, and piezo

micromanipulation [135–140]. It has been proposed that such techniques not only aid in mechanical hatching but could also enhance direct transport of nutrients from incubating media by allowing for an easier two-way exchange of metabolites [141].

Early prospective randomized control trials undertaken at our centre suggested maximal benefit from assisted hatching in individuals over the age of 38, and specifically for patients with thickened zonae [135]. Subsequent studies examining the routine or targeted implementation of assisted hatching in IVF cycles have shown mixed results. While the data has not convincingly shown that universal application of assisted hatching is beneficial, individuals with repeated implantation failure may preferentially benefit from the technique [138, 142, 143]. Stein et al. reported that partial zona dissection resulted in a significant improvement in implantation and pregnancy rates in women older than age 38 who had a history of RIF [144]. Petersen et al. similarly reported higher implantation rates when embryos underwent laser-assisted zona thinning, but only in individuals with at least two prior implantation failures [145]. Magli et al. conducted a randomized controlled trial which included women who were >38 years old (45 cycles), patients with three or more prior failed IVF attempts (70 cycles), and patients that met both criteria (20 cycles). Clinical pregnancies per cycle were significantly higher in patients undergoing assisted hatching where either age (31% vs 10%) or repeated failure (36% vs 17%) was the indication [146]. Corroborating these reports, a meta-analyses examining data from five randomized controlled trials (561 patients) revealed a 73% improvement in clinical pregnancy (RR 1.73; 95% CI 1.37–2.17) when assisted hatching was employed in individuals with RIF [147]. Unselected patients, however, do not seem to experience the same benefit [148].

The optimal technique for assisted hatching remains controversial. Hsieh et al. reported that hatching with a diode laser provided greater benefit than chemical assisted hatching in older patients [149]. Primi et al. similarly showed better results for patients with RIF when employing the diode laser [150]. Conversely, Balaban et al. did not discern any appreciable difference when examining partial zona dissection, acid Tyrodes, diode laser, or pronase thinning [151]. Others have argued that the optimal implementation of assisted hatching involves laser assisted thinning of the zona, without a complete breach, limiting the procedure to only a quarter of the circumference of the embryo [152]. Given the heterogeneity of techniques and the wide range of published evidence, no specific assisted hatching technique has been established as the gold standard for patients with repeated implantation failure.

Endometrial “scratch”

Significant controversy exists over the benefit of “endometrial scratch” as a method for fostering implantation, and the technique has fallen out of favour since a 2019 randomized controlled trial failed to show a benefit of the technique [153]. The method is purported to induce a “healing process” that allows for release of cytokines and other growth factors, which facilitate implantation. Mechanical endometrial injury prior to controlled ovarian hyperstimulation has been proposed as a method to induce decidualization and attract cytokines, growth factors, LIF, and other immune modulators in the endometrium [154, 155]. Barash et al. in 2003 first suggested an association between endometrial biopsy and implantation in a study of 134 good responders who failed to conceive in one or more prior IVF cycles with at least three embryos transferred [156]. Fifty-four of 134 subjects were

subjected to repeated endometrial biopsy on days 8, 12, 21, and 26 of the cycle preceding IVE, with the data suggesting a significant improvement in subsequent implantation rates (27.7% vs 14.3%) following repeated biopsies. Subsequent randomized controlled trials have employed a variety of inclusion criteria and frequency/timing of biopsies, and have suggested, overall, that there is an implantation benefit following the intervention [157–159].

Whereas initial studies seemed promising, subsequent data has been mixed, with some studies revealing a decrease in pregnancy rates in women undergoing biopsies prior to IVF [157, 158, 160–162]. In a small randomized controlled trial of women with RIF involving a sham cervical biopsy for the control group, clinical pregnancy and live-birth rates were lower in the experimental group [162]. Two additional studies suggested no benefit of endometrial scratch in unselected populations undergoing IVF [160, 163]. In a sub-analysis of our own autologous endometrial co-culture program at Cornell, no improvement in implantation was seen for those patients having a co-culture biopsy in the luteal phase immediately preceding the IVF cycle [164]. Similarly, in another study, endometrial disruption in 39 patients with a history of failed euploid embryo transfer did not improve implantation as compared to 251 control patients who did not undergo endometrial biopsies [165].

In 2019, a well-designed multicentre trial from New Zealand randomized 1364 patients to either endometrial scratching by pipelle biopsy prior to cycle initiation versus no intervention. In this study, there were no differences in live-birth rates between the endometrial scratch group and the control group, and in fact the rates were identical (26.1%) [153]. A subsequent randomized control trial from the Netherlands randomized 933 patients with one previous failed IVF cycle, and again failed to reveal a benefit to endometrial scratch, although pregnancy rates were slightly higher in the intervention arm (23.7% live birth vs 19.1% live birth, respectively) [166]. Given these latest randomized controlled trials, significant questions remain regarding the purported benefit of the procedure, optimal frequency of biopsy if performed, suitable timing of sampling, and what, if any, harm might exist [167]. In the wake of RCT data showing no benefit, endometrial scratch should not be recommended as a first-line intervention for RIF patients without careful counselling about recent data calling the practice into question.

Co-culture

The *in vitro* culture conditions in mammalian IVF attempt to simulate *in vivo* conditions, but growth, biochemical synthetic activity, and reproductive competence may fall short during *in vitro* development. Co-culture of *in vitro* derived embryos with either tubal epithelium, endometrial epithelium, granulosa, or cumulus cells has been proposed to foster more supportive culture conditions [168–172]. Variable reported success rates with these techniques are likely attributable, at least in part, to differences in cell lines, maintenance of cells, and various environmental factors within each laboratory.

Use of Vero cells (from monkey kidney epithelium) and bovine oviductal epithelium have both been noted to improve embryo quality and pregnancy rates in poor prognosis patients [173, 174]. Co-culture of human embryos with buffalo rat liver cells also suggested a favourable trend (34% vs 28%) towards improved pregnancy rates in patients with prior failures [175]. Xeno-culture, however, poses both theoretical and practical infectious risks that make the use of various animal cells less than ideal for human embryos.

Because of these potential risks, investigators have focused on utilizing either homologous or autologous human cells in co-culture systems. Tubal cells from the ampullary portion of the fallopian tube have been harvested during hysterectomies or tubal ligations and passaged several times to allow for use in multiple patients [168, 176]. Embryonic viability, morphological appearance, and number of blastomeres were enhanced when tubal epithelial co-culture was employed, with a second study revealing higher pregnancy, implantation, and embryo cryopreservation rates [168, 176]. However, the risk of transmission of infectious agents along with Creutzfeldt-Jakob disease limits the desirability of homologous techniques.

At the Center for Reproductive Medicine at Weill Cornell Medical College, we have developed and successfully applied a unique co-culture system that uses the patient's own endometrial cells to enhance embryo development [177, 178]. Patients undergo an endometrial biopsy in the mid-luteal phase of a cycle preceding their IVF treatment cycle, and endometrial glandular epithelial and stromal cells are separated by differential sedimentation and plated until a mono-layer is achieved. The cells are then frozen and later thawed during the patient's treatment cycle. An equal mixture of glandular epithelial and stromal cells is seeded into a four-well tissue plate containing Ham's F-10 medium supplemented with 15% patient serum. Embryos are introduced into the co-culture system after fertilization and maintained with the autologous endometrial cells until the day of transfer.

Human endometrial co-culture has been noted to be beneficial to blastocyst development, presumably owing to a chemical cross-talk and paracrine signalling between embryo and endometrium [179–181]. The use of autologous endometrial cells for co-culture in patients with repeated implantation failure was first reported by Jayot et al. in 1995, with a pregnancy rate of 21% as compared to 8% in patients' previous cycles [182]. Nieto used predominantly endometrial epithelial cells and reported a decrease in fragmentation among day 3 embryos [169]. Simon et al. achieved a 39.2% blastocyst formation rate, an 11.8% implantation rate, and a 20.2% pregnancy rate with an autologous endometrial co-culture system in 168 cycles among patients with three or more failed implantation cycles [183]. Eyheremendy et al. similarly demonstrated benefit utilizing autologous endometrial cell co-culture with day 3 transfer in patients with RIF [184].

In our own experience, sibling oocytes from RIF patients undergoing endometrial co-culture exhibit lower fragmentation and more blastomeres at the time of transfer as compared to traditionally cultured embryos [177]. Further published studies revealed implantation and clinical pregnancy rates of 15% and 29%, respectively, in patients with prior IVF failures associated with poor embryo quality [178]. We observe the best results when biopsies are performed in the mid to late luteal phase as opposed to the early luteal phase of the menstrual cycle [185]. A meta-analysis of 17 studies has suggested an improvement in blastomere number, implantation, and pregnancy rates with the utilization of co-culture [186]. While the data suggests a distinct benefit to autologous endometrial co-culture for patients with RIF, such programs are difficult to maintain given the resources in personnel and time required. Moreover, as potentially better embryo incubation techniques such as time-lapse microscopy at low oxygen tensions emerge, the incremental benefit of endometrial co-culture remains to be further defined for these difficult repeated implantation failure patients.

Conclusion

Although treatment of patients with a history of repeated implantation failure can be discouraging, techniques and methodologies striving to optimize IVF success in these patients continue to evolve. We must continue to investigate and elucidate factors that may prevent our patients from achieving live births. For this subset of challenging patients, there will always exist a cycle of new ideas, investigation, and validation to know what will benefit them. Current experiments include intrauterine infusion of peripheral blood mononuclear cells, platelet-rich plasma, or subcutaneously injected granulocyte colony-stimulating factor. These are all based on recruitment of lymphocytes, growth factors, and T cells into the endometrium. As in past experience of unproven treatments, one should exercise caution to minimize risk of undue exposure to patients in our efforts to improve live birth rates. Further evaluation of embryo–endometrial cross-talk, and the ideal timing of transfer into a receptive endometrium, may lead to new treatments for patients experiencing RIF. Likewise, improved embryo culture and embryo analytic techniques may offer finer discernment of embryos with the greatest implantation potential. The physician caring for a patient or couple with RIF must carefully review the prior diagnostic workup, complete the investigation with appropriate analytic techniques, and offer empathy and encouragement while providing accurate counsel on the likelihood of success.

References

1. <https://archive.cdc.gov/#/details?url=https://www.cdc.gov/art/reports/2019/pdf/2019-Report-ART-Fertility-Clinic-National-Summary-h.pdf>
2. Steiner AZ, et al. Association between biomarkers of ovarian reserve and infertility among older women of reproductive age. *JAMA*. 2017;318:1367–76. doi: [10.1001/jama.2017.14588](https://doi.org/10.1001/jama.2017.14588).
3. Reig A, Fransasiak J, Scott RT Jr., Seli E. The impact of age beyond ploidy: Outcome data from 8175 euploid single embryo transfers. *J Assist Reprod Genet*. 2020;37:595–602. doi: [10.1007/s10815-020-01739-0](https://doi.org/10.1007/s10815-020-01739-0).
4. Shaulov T, Sierra S, Sylvestre C. Recurrent implantation failure in IVF: A Canadian fertility and andrology society clinical practice guideline. *Reprod Biomed Online*. 2020;41:819–33. doi: [10.1016/j.rbmo.2020.08.007](https://doi.org/10.1016/j.rbmo.2020.08.007).
5. Pirtea P, et al. Rate of true recurrent implantation failure is low: Results of three successive frozen euploid single embryo transfers. *Fertil Steril*. 2021;115:45–53. doi: [10.1016/j.fertnstert.2020.07.002](https://doi.org/10.1016/j.fertnstert.2020.07.002).
6. Cimadomo D, Craciunas L, Vermeulen N, Vomstein K, Toth B. Definition, diagnostic and therapeutic options in recurrent implantation failure: An international survey of clinicians and embryologists. *Hum Reprod*. 2021;36:305–17. doi: [10.1093/humrep/deaa317](https://doi.org/10.1093/humrep/deaa317).
7. Stern C, Pertile M, Norris H, Hale L, Baker HW. Chromosome translocations in couples with in-vitro fertilization implantation failure. *Hum Reprod*. 1999;14:2097–101.
8. Raziel A, et al. Increased frequency of female partner chromosomal abnormalities in patients with high-order implantation failure after in vitro fertilization. *Fertil Steril*. 2002;78:515–9.
9. De Sutter P, Stadhouders R, Dutre M, Gerris J, Dhont M. Prevalence of chromosomal abnormalities and timing of karyotype analysis in patients with recurrent implantation failure (RIF) following assisted reproduction. *Facts Views Vis Obgyn*. 2012;4:59–65.
10. Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: A comparison of several species. *Mol Reprod Dev*. 1990;26:90–100. doi: [10.1002/mrd.1080260113](https://doi.org/10.1002/mrd.1080260113).

11. Demko ZP, Simon AL, McCoy RC, Petrov DA, Rabinowitz M. Effects of maternal age on euploidy rates in a large cohort of embryos analyzed with 24-chromosome single-nucleotide polymorphism-based preimplantation genetic screening. *Fertil Steril.* 2016;105:1307–13. doi: [10.1016/j.fertnstert.2016.01.025](https://doi.org/10.1016/j.fertnstert.2016.01.025).
12. Fransasiak JM, et al. The nature of aneuploidy with increasing age of the female partner: A review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertil Steril.* 2014;101:656–63.e651. doi: [10.1016/j.fertnstert.2013.11.004](https://doi.org/10.1016/j.fertnstert.2013.11.004).
13. Rubio C, et al. Clinical application of embryo aneuploidy testing by next-generation sequencing. *Biol Reprod.* 2019;101:1083–1090. doi: [10.1093/biolre/ioz019](https://doi.org/10.1093/biolre/ioz019).
14. Pehlivan T, et al. Impact of preimplantation genetic diagnosis on IVF outcome in implantation failure patients. *Reprod Biomed Online.* 2003;6:232–7.
15. Gianaroli L, et al. Will preimplantation genetic diagnosis assist patients with a poor prognosis to achieve pregnancy? *Hum Reprod.* 1997;12:1762–7.
16. Greco E, et al. Comparative genomic hybridization selection of blastocysts for repeated implantation failure treatment: A pilot study. *Biomed Res Int.* 2014;2014:457913. doi: [10.1155/2014/457913](https://doi.org/10.1155/2014/457913).
17. Sato T, et al. Preimplantation genetic testing for aneuploidy: A comparison of live birth rates in patients with recurrent pregnancy loss due to embryonic aneuploidy or recurrent implantation failure. *Hum Reprod.* 2019;34:2340–8. doi: [10.1093/humrep/dez229](https://doi.org/10.1093/humrep/dez229).
18. Blockeel C, et al. Prospectively randomized controlled trial of PGS in IVF/ICSI patients with poor implantation. *Reprod Biomed Online.* 2008;17:848–54.
19. Treff NR, et al. Development and validation of an accurate quantitative real-time polymerase chain reaction-based assay for human blastocyst comprehensive chromosomal aneuploidy screening. *Fertil Steril.* 2012;97:819–24. doi: [10.1016/j.fertnstert.2012.01.115](https://doi.org/10.1016/j.fertnstert.2012.01.115).
20. Fragouli E, et al. Comprehensive chromosome screening of polar bodies and blastocysts from couples experiencing repeated implantation failure. *Fertil Steril.* 2010;94:875–87. doi: [10.1016/j.fertnstert.2009.04.053](https://doi.org/10.1016/j.fertnstert.2009.04.053).
21. Lee HL, et al. In vitro fertilization with preimplantation genetic screening improves implantation and live birth in women age 40 through 43. *J Assist Reprod Genet.* 2015;32:435–44. doi: [10.1007/s10815-014-0417-7](https://doi.org/10.1007/s10815-014-0417-7).
22. Munne S, et al. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: A multicenter randomized clinical trial. *Fertil Steril.* 2019;112:1071–79.e1077. doi: [10.1016/j.fertnstert.2019.07.1346](https://doi.org/10.1016/j.fertnstert.2019.07.1346).
23. Yan J, et al. Live birth with or without preimplantation genetic testing for aneuploidy. *N Engl J Med.* 2021;385:2047–58. doi: [10.1056/NEJMoa2103613](https://doi.org/10.1056/NEJMoa2103613).
24. Robinson L, et al. The effect of sperm DNA fragmentation on miscarriage rates: A systematic review and meta-analysis. *Hum Reprod.* 2012;27:2908–17. doi: [10.1093/humrep/des261](https://doi.org/10.1093/humrep/des261).
25. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl.* 2002;23:25–43.
26. Bungum M, et al. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod.* 2004;19:1401–8. doi: [10.1093/humrep/deh280](https://doi.org/10.1093/humrep/deh280).
27. Bungum M, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod.* 2007;22:174–9. doi: [10.1093/humrep/del326](https://doi.org/10.1093/humrep/del326).
28. Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: A prospective cohort study. *Hum Reprod.* 2002;17:3122–8.
29. Muriel L, et al. Value of the sperm chromatin dispersion test in predicting pregnancy outcome in intrauterine insemination: A blind prospective study. *Hum Reprod.* 2006;21:738–44. doi: [10.1093/humrep/dei403](https://doi.org/10.1093/humrep/dei403).
30. Absalan F, et al. Value of sperm chromatin dispersion test in couples with unexplained recurrent abortion. *J Assist Reprod Genet.* 2012;29:11–14. doi: [10.1007/s10815-011-9647-0](https://doi.org/10.1007/s10815-011-9647-0).
31. Brahem S, et al. Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. *Urology.* 2011;78:792–6. doi: [10.1016/j.urology.2011.05.049](https://doi.org/10.1016/j.urology.2011.05.049).
32. Greco E, et al. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. *Hum Reprod.* 2005;20:226–30. doi: [10.1093/humrep/deh590](https://doi.org/10.1093/humrep/deh590).
33. Steele EK, McClure N, Maxwell RJ, Lewis SE. A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. *Mol Hum Reprod.* 1999;5:831–5.
34. Suganuma R, Yanagimachi R, Meistrich ML. Decline in fertility of mouse sperm with abnormal chromatin during epididymal passage as revealed by ICSI. *Hum Reprod.* 2005;20:3101–8. doi: [10.1093/humrep/dei169](https://doi.org/10.1093/humrep/dei169).
35. Weissman A, et al. Pregnancies and live births following ICSI with testicular spermatozoa after repeated implantation failure using ejaculated spermatozoa. *Reprod Biomed Online.* 2008;17:605–9.
36. Coughlan C, et al. Sperm DNA fragmentation, recurrent implantation failure and recurrent miscarriage. *Asian J Androl.* 2015;17:681–5. doi: [10.4103/1008-682X.144946](https://doi.org/10.4103/1008-682X.144946).
37. Best JC, et al. Elevated sperm DNA fragmentation does not predict recurrent implantation failure. *Andrologia.* 2021;53:e14094. doi: [10.1111/and.14094](https://doi.org/10.1111/and.14094).
38. Jakab A, et al. Intracytoplasmic sperm injection: A novel selection method for sperm with normal frequency of chromosomal aneuploidies. *Fertil Steril.* 2005;84:1665–73. doi: [10.1016/j.fertnstert.2005.05.068](https://doi.org/10.1016/j.fertnstert.2005.05.068).
39. Said TM, et al. Advantage of combining magnetic cell separation with sperm preparation techniques. *Reprod Biomed Online.* 2005;10:740–6.
40. Sakkas D, Alvarez JG. Sperm DNA fragmentation: Mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril.* 2010;93:1027–36. doi: [10.1016/j.fertnstert.2009.10.046](https://doi.org/10.1016/j.fertnstert.2009.10.046).
41. Bartoov B, et al. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. *Fertil Steril.* 2003;80:1413–9.
42. Berkovitz A, et al. How to improve IVF-ICSI outcome by sperm selection. *Reprod Biomed Online.* 2006;12:634–8.
43. Nadalini M, Tarozzi N, Distratis V, Scaravelli G, Borini A. Impact of intracytoplasmic morphologically selected sperm injection on assisted reproduction outcome: A review. *Reprod Biomed Online.* 2009;19(Suppl 3):45–55.
44. Oliveira JB, et al. Pregnancy outcomes in women with repeated implantation failures after intracytoplasmic morphologically selected sperm injection (IMSI). *Reprod Biol Endocrinol.* 2011;9:99. doi: [10.1186/1477-7827-9-99](https://doi.org/10.1186/1477-7827-9-99).
45. Franco JG Jr., et al. Significance of large nuclear vacuoles in human spermatozoa: Implications for ICSI. *Reprod Biomed Online.* 2008;17:42–5.
46. Oliveira JB, et al. Correlation between semen analysis by motile sperm organelle morphology examination and sperm DNA damage. *Fertil Steril.* 2010;94:1937–40. doi: [10.1016/j.fertnstert.2010.01.042](https://doi.org/10.1016/j.fertnstert.2010.01.042).
47. Perdrix A, et al. Assessment of acrosome and nuclear abnormalities in human spermatozoa with large vacuoles. *Hum Reprod.* 2011;26:47–58. doi: [10.1093/humrep/deq297](https://doi.org/10.1093/humrep/deq297).
48. Boitrelle F, et al. Large human sperm vacuoles observed in motile spermatozoa under high magnification: Nuclear thumbprints linked to failure of chromatin condensation. *Hum Reprod.* 2011;26:1650–58. doi: [10.1093/humrep/der129](https://doi.org/10.1093/humrep/der129).

49. El Khattabi L, et al. Is intracytoplasmic morphologically selected sperm injection effective in patients with infertility related to teratozoospermia or repeated implantation failure? *Fertil Steril.* 2013;100:62–8. doi: [10.1016/j.fertnstert.2013.02.048](https://doi.org/10.1016/j.fertnstert.2013.02.048).
50. Burrello N, et al. Lower sperm aneuploidy frequency is associated with high pregnancy rates in ICSI programmes. *Hum Reprod.* 2003;18:1371–6.
51. Rodrigo L, et al. Sperm chromosomal abnormalities and their contribution to human embryo aneuploidy. *Biol Reprod.* 2019; 101:1091–101. doi: [10.1093/biolre/izz125](https://doi.org/10.1093/biolre/izz125).
52. Makrakis E, Pantos K. The outcomes of hysteroscopy in women with implantation failures after in-vitro fertilization: Findings and effect on subsequent pregnancy rates. *Curr Opin Obstet Gynecol.* 2010;22:339–43. doi: [10.1097/GCO.0b013e32833beaa3](https://doi.org/10.1097/GCO.0b013e32833beaa3).
53. Buttram VC Jr., Reiter R. Uterine leiomyomata: Etiology, symptomatology, and management. *Fertil Steril.* 1981;36:433–45.
54. Donnez J, Jadoul P. What are the implications of myomas on fertility? A need for a debate? *Hum Reprod.* 2002;17:1424–1430.
55. Taylor E, Gomel V. The uterus and fertility. *Fertil Steril.* 2008;89:1–16. doi: [10.1016/j.fertnstert.2007.09.069](https://doi.org/10.1016/j.fertnstert.2007.09.069).
56. Metwally M, Farquhar CM, Li TC. Is another meta-analysis on the effects of intramural fibroids on reproductive outcomes needed? *Reprod Biomed Online.* 2011;23:2–14. doi: [10.1016/j.rbmo.2010.08.006](https://doi.org/10.1016/j.rbmo.2010.08.006).
57. Pritts EA, Parker WH, Olive DL. Fibroids and infertility: An updated systematic review of the evidence. *Fertil Steril.* 2009;91: 1215–23. doi: [10.1016/j.fertnstert.2008.01.051](https://doi.org/10.1016/j.fertnstert.2008.01.051).
58. Sunkara SK, Khairy M, El-Toukhy T, Khalaf Y, Coomarasamy A. The effect of intramural fibroids without uterine cavity involvement on the outcome of IVF treatment: A systematic review and meta-analysis. *Hum Reprod.* 2010;25:418–29. doi: [10.1093/humrep/dep396](https://doi.org/10.1093/humrep/dep396).
59. Taylor HS. Fibroids: When should they be removed to improve in vitro fertilization success? *Fertil Steril.* 2018;109:784–5. doi: [10.1016/j.fertnstert.2018.03.003](https://doi.org/10.1016/j.fertnstert.2018.03.003).
60. Richlin SS, Ramachandran S, Shanti A, Murphy AA, Parthasarathy S. Glycodelin levels in uterine flushings and in plasma of patients with leiomyomas and polyps: Implications for implantation. *Hum Reprod.* 2002;17:2742–7.
61. Shokeir TA, Shalan HM, El-Shafei MM. Significance of endometrial polyps detected hysteroscopically in eumenorrheic infertile women. *J Obstet Gynaecol Res.* 2004;30:84–9.
62. Spiewankiewicz B, et al. The effectiveness of hysteroscopic polypectomy in cases of female infertility. *Clin Exp Obstet Gynecol.* 2003;30:23–5.
63. Varasteh NN, Neuwirth RS, Levin B, Keltz MD. Pregnancy rates after hysteroscopic polypectomy and myomectomy in infertile women. *Obstet Gynecol.* 1999;94:168–71.
64. Perez-Medina T, et al. Endometrial polyps and their implication in the pregnancy rates of patients undergoing intrauterine insemination: A prospective, randomized study. *Hum Reprod.* 2005;20 :1632–35. doi: [10.1093/humrep/deh822](https://doi.org/10.1093/humrep/deh822).
65. Dawood A, Al-Talib A, Tulandi T. Predisposing factors and treatment outcome of different stages of intrauterine adhesions. *J Obstet Gynaecol Can.* 2010;32:767–770.
66. Katz Z, Ben-Arie A, Lurie S, Manor M, Insler V. Reproductive outcome following hysteroscopic adhesiolysis in Asherman's syndrome. *Int J Fertil Menopausal Stud.* 1996;41:462–5.
67. Pace S, Stentella P, Catania R, Palazzetti PL, Frega A. Endoscopic treatment of intrauterine adhesions. *Clin Exp Obstet Gynecol.* 2003;30:26–8.
68. Yasmin H, Nasir A, Noorani KJ. Hysteroscopic management of Ashermans syndrome. *J Pak Med Assoc.* 2007;57:553–5.
69. Zikopoulos KA, et al. Live delivery rates in subfertile women with Asherman's syndrome after hysteroscopic adhesiolysis using the resectoscope or the Versapoint system. *Reprod Biomed Online.* 2004;8:720–5.
70. Wang WJ, et al. Endometrial TGF-beta, IL-10, IL-17 and autophagy are dysregulated in women with recurrent implantation failure with chronic endometritis. *Reprod Biol Endocrinol.* 2019;17:2. doi: [10.1186/s12958-018-0444-9](https://doi.org/10.1186/s12958-018-0444-9).
71. Li Y, et al. Evaluation of peripheral and uterine immune status of chronic endometritis in patients with recurrent reproductive failure. *Fertil Steril.* 2020;113:187–96.e181. doi: [10.1016/j.fertnstert.2019.09.001](https://doi.org/10.1016/j.fertnstert.2019.09.001).
72. Strandell A, Waldenstrom U, Nilsson L, Hamberger L. Hydro-salpinx reduces in-vitro fertilization/embryo transfer pregnancy rates. *Hum Reprod.* 1994;9:861–3.
73. Feghali J, et al. Systematic hysteroscopy prior to in vitro fertilization. *Gynecol Obstet Fertil.* 2003;31:127–31.
74. Johnston-MacAnanny EB, et al. Chronic endometritis is a frequent finding in women with recurrent implantation failure after in vitro fertilization. *Fertil Steril.* 2010;93:437–41. doi: [10.1016/j.fertnstert.2008.12.131](https://doi.org/10.1016/j.fertnstert.2008.12.131).
75. Cincinelli E, et al. Prevalence of chronic endometritis in repeated unexplained implantation failure and the IVF success rate after antibiotic therapy. *Hum Reprod.* 2015;30:323–30. doi: [10.1093/humrep/deu292](https://doi.org/10.1093/humrep/deu292).
76. Tagliano A, et al. Effects of chronic endometritis therapy on in vitro fertilization outcome in women with repeated implantation failure: A systematic review and meta-analysis. *Fertil Steril.* 2018;110:103–12.e101. doi: [10.1016/j.fertnstert.2018.03.017](https://doi.org/10.1016/j.fertnstert.2018.03.017).
77. Xiong Y, et al. Impact of oral antibiotic treatment for chronic endometritis on pregnancy outcomes in the following frozen-thawed embryo transfer cycles of infertile women: A cohort study of 640 embryo transfer cycles. *Fertil Steril.* 2021;116:413–21. doi: [10.1016/j.fertnstert.2021.03.036](https://doi.org/10.1016/j.fertnstert.2021.03.036).
78. Tremellen K, Russell P. Adenomyosis is a potential cause of recurrent implantation failure during IVF treatment. *Aus N Z J Obstet Gynaecol.* 2011;51:280–3. doi: [10.1111/j.1479-828X.2010.01276.x](https://doi.org/10.1111/j.1479-828X.2010.01276.x).
79. Puente JM, et al. Adenomyosis in infertile women: Prevalence and the role of 3D ultrasound as a marker of severity of the disease. *Reprod Biol Endocrinol.* 2016;14:60. doi: [10.1186/s12958-016-0185-6](https://doi.org/10.1186/s12958-016-0185-6).
80. Younes G, Tulandi T. Effects of adenomyosis on in vitro fertilization treatment outcomes: A meta-analysis. *Fertil Steril.* 2017;108, 483–490.e483. doi: [10.1016/j.fertnstert.2017.06.025](https://doi.org/10.1016/j.fertnstert.2017.06.025).
81. Horton J, et al. Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: A systematic review and meta-analysis. *Hum Reprod Update.* 2019;25:592–632. doi: [10.1093/humupd/dmz012](https://doi.org/10.1093/humupd/dmz012).
82. Maheshwari A, Gurunath S, Fatima F, Bhattacharya S. Adenomyosis and subfertility: A systematic review of prevalence, diagnosis, treatment and fertility outcomes. *Hum Reprod Update.* 2012;18:374–92. doi: [10.1093/humupd/dms006](https://doi.org/10.1093/humupd/dms006).
83. Sunkara SK, Khan KS. Adenomyosis and female fertility: A critical review of the evidence. *J Obstet Gynaecol.* 2012;32:113–6. doi: [10.3109/01443615.2011.624208](https://doi.org/10.3109/01443615.2011.624208).
84. Nirgianakis K, et al. Fertility, pregnancy and neonatal outcomes of patients with adenomyosis: A systematic review and meta-analysis. *Reprod Biomed Online.* 2021;42:185–206. doi: [10.1016/j.rbmo.2020.09.023](https://doi.org/10.1016/j.rbmo.2020.09.023).
85. Demirogl A, Gurgan T. Effect of treatment of intrauterine pathologies with office hysteroscopy in patients with recurrent IVF failure. *Reprod Biomed Online.* 2004;8:590–4.
86. Rama Raju GA, Shashi Kumari G, Krishna KM, Prakash GJ, Madan K. Assessment of uterine cavity by hysteroscopy in assisted reproduction programme and its influence on pregnancy outcome. *Archives of Gynecology and Obstetrics.* 2006;274:160–4, doi: [10.1007/s00404-006-0174-7](https://doi.org/10.1007/s00404-006-0174-7).
87. Gao M, Sun Y, Xie H, Fang S, Zhao X. Hysteroscopy prior to repeat embryo transfer may improve pregnancy outcomes for asymptomatic women with repeated implantation failure. *J Obstet Gynaecol Res.* 2015;41:1569–76. doi: [10.1111/jog.12773](https://doi.org/10.1111/jog.12773).

88. Hosseini MA, et al. Hysteroscopy in patients with repeated implantation failure improves the outcome of assisted reproductive technology in fresh and frozen cycles. *J Obstet Gynaecol Res.* 2014;40:1324–30. doi: [10.1111/jog.12315](https://doi.org/10.1111/jog.12315).
89. Lambert M, et al. [Repeated in vitro fertilization failure: Abnormalities identified in the diagnostic assessment]. *Gynecol Obstet Fertil.* 2016;44:565–71. doi: [10.1016/j.gyobfe.2016.08.006](https://doi.org/10.1016/j.gyobfe.2016.08.006).
90. Pabuccu EG, Yalcin I, Bodur T, Caglar GS, Pabuccu R. Impact of office hysteroscopy in repeated implantation failure: Experience of a single center. *J Turk Ger Gynecol Assoc.* 2016;17:197–200. doi: [10.5152/jtggta.2016.16166](https://doi.org/10.5152/jtggta.2016.16166).
91. Shokeir T, Abdelshaheed M. Sonohysterography as a first-line evaluation for uterine abnormalities in women with recurrent failed in vitro fertilization-embryo transfer. *Fertil Steril.* 2009;91:1321–22. doi: [10.1016/j.fertnstert.2008.02.135](https://doi.org/10.1016/j.fertnstert.2008.02.135).
92. Soares SR, Barbosa dos Reis MM, Camargos AF. Diagnostic accuracy of sonohysterography, transvaginal sonography, and hysterosalpingography in patients with uterine cavity diseases. *Fertil Steril.* 2000;73:406–11.
93. Grimbizis GF, et al. A prospective comparison of transvaginal ultrasound, saline infusion sonohysterography, and diagnostic hysteroscopy in the evaluation of endometrial pathology. *Fertil Steril.* 2010;94:2720–25. doi: [10.1016/j.fertnstert.2010.03.047](https://doi.org/10.1016/j.fertnstert.2010.03.047).
94. El-Toukhy T, et al. Hysteroscopy in recurrent in-vitro fertilisation failure (TROPHY): A multicentre, randomised controlled trial. *Lancet.* 2016;387:2614–21. doi: [10.1016/S0140-6736\(16\)00258-0](https://doi.org/10.1016/S0140-6736(16)00258-0).
95. Galgani M, et al. Regulatory t cells, inflammation, and endoplasmic reticulum stress in women with defective endometrial receptivity. *Fertil Steril.* 2015;103:1579–86.e1571. doi: [10.1016/j.fertnstert.2015.03.014](https://doi.org/10.1016/j.fertnstert.2015.03.014).
96. Achache H, Tsafir A, Prus D, Reich R, Revel A. Defective endometrial prostaglandin synthesis identified in patients with repeated implantation failure undergoing in vitro fertilization. *Fertil Steril.* 2010;94:1271–8. doi: [10.1016/j.fertnstert.2009.07.1668](https://doi.org/10.1016/j.fertnstert.2009.07.1668).
97. Riesewijk A, et al. Gene Expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Mol Hum Reprod.* 2003;9:253–64.
98. Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA. Molecular classification of human endometrial cycle stages by transcriptional profiling. *Mol Hum Reprod.* 2004;10:879–93. doi: [10.1093/molehr/gah121](https://doi.org/10.1093/molehr/gah121).
99. Talbi S, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology.* 2006;147:1097–121. doi: [10.1210/en.2005-1076](https://doi.org/10.1210/en.2005-1076).
100. Horcajadas JA, Pellicer A, Simon C. Wide genomic analysis of human endometrial receptivity: New times, new opportunities. *Hum Reprod Update.* 2007;13:77–86. doi: [10.1093/humupd/dml046](https://doi.org/10.1093/humupd/dml046).
101. Ruiz-Alonso M, Blesa D, Simon C. The genomics of the human endometrium. *Biochim Biophys Acta.* 2012;1822:1931–42. doi: [10.1016/j.bbapap.2012.05.004](https://doi.org/10.1016/j.bbapap.2012.05.004).
102. Koler M, et al. Disrupted gene pattern in patients with repeated in vitro fertilization (IVF) failure. *Hum Reprod.* 2009;24:2541–8. doi: [10.1093/humrep/dep193](https://doi.org/10.1093/humrep/dep193).
103. Bersinger NA, Wunder DM, Birkhauser MH, Mueller MD. Gene Expression in cultured endometrium from women with different outcomes following IVF. *Mol Hum Reprod.* 2008;14:475–84. doi: [10.1093/molehr/gan036](https://doi.org/10.1093/molehr/gan036).
104. Tapia A, et al. Differences in the endometrial transcript profile during the receptive period between women who were refractory to implantation and those who achieved pregnancy. *Hum Reprod.* 2008;23:340–51. doi: [10.1093/humrep/dem319](https://doi.org/10.1093/humrep/dem319).
105. Diaz-Gimeno P, et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril.* 2011;95:50–60. 60 e51–15. doi: [10.1016/j.fertnstert.2010.04.063](https://doi.org/10.1016/j.fertnstert.2010.04.063).
106. Diaz-Gimeno P, et al. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. *Fertil Steril.* 2013;99:508–17. doi: [10.1016/j.fertnstert.2012.09.046](https://doi.org/10.1016/j.fertnstert.2012.09.046).
107. Rosenwaks Z. Donor eggs: Their application in modern reproductive technologies. *Fertil Steril.* 1987;47:895–909. doi: [10.1016/s0015-0282\(16\)59220-6](https://doi.org/10.1016/s0015-0282(16)59220-6).
108. Tan J, et al. The role of the endometrial receptivity array (ERA) in patients who have failed euploid embryo transfers. *J Assist Reprod Genet.* 2018;35:683–92. doi: [10.1007/s10815-017-1112-2](https://doi.org/10.1007/s10815-017-1112-2).
109. Ruiz-Alonso M, et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. *Fertil Steril.* 2013;100:818–24. doi: [10.1016/j.fertnstert.2013.05.004](https://doi.org/10.1016/j.fertnstert.2013.05.004).
110. Hashimoto T, et al. Efficacy of the endometrial receptivity array for repeated implantation failure in Japan: A retrospective, two-centers study. *Reprod Med Biol.* 2017;16:290–6. doi: [10.1002/rmb2.12041](https://doi.org/10.1002/rmb2.12041).
111. Simon C, et al. A 5-year multicentre randomized controlled trial comparing personalized, frozen and fresh blastocyst transfer in IVF. *Reprod Biomed Online.* 2020;41:402–15. doi: [10.1016/j.rbmo.2020.06.002](https://doi.org/10.1016/j.rbmo.2020.06.002).
112. Lensen S, Wilkinson J, van Wely M, Farquhar C. Comments on the methodology of an endometrial receptivity array trial. *Reprod Biomed Online.* 2021;42:283. doi: [10.1016/j.rbmo.2020.09.027](https://doi.org/10.1016/j.rbmo.2020.09.027).
113. Cozzolino M, Diaz-Gimeno P, Pellicer A, Garrido N. Evaluation of the endometrial receptivity assay and the preimplantation genetic test for aneuploidy in overcoming recurrent implantation failure. *J Assist Reprod Genet.* 2020;37:2989–97. doi: [10.1007/s10815-020-01948-7](https://doi.org/10.1007/s10815-020-01948-7).
114. Riestenberg C, Kroener L, Quinn M, Ching K, Ambartsumyan G. Routine endometrial receptivity array in first embryo transfer cycles does not improve live birth rate. *Fertil Steril.* 2021;115:1001–6. doi: [10.1016/j.fertnstert.2020.09.140](https://doi.org/10.1016/j.fertnstert.2020.09.140).
115. Ben Rafael Z. Endometrial receptivity analysis (ERA) test: An unproven technology. *Hum Reprod Open.* 2021;2021:hoab010. doi: [10.1093/hropen/hoab010](https://doi.org/10.1093/hropen/hoab010).
116. Coughlan C, et al. Recurrent implantation failure: Definition and management. *Reprod Biomed Online.* 2014;28:14–38. doi: [10.1016/j.rbmo.2013.08.011](https://doi.org/10.1016/j.rbmo.2013.08.011).
117. Camus E, et al. Pregnancy rates after in-vitro fertilization in cases of tubal infertility with and without hydrosalpinx: A meta-analysis of published comparative studies. *Hum Reprod.* 1999;14:1243–9.
118. Strandell A, et al. Hydrosalpinx and IVF outcome: A prospective, randomized multicentre trial in scandinavia on salpingectomy prior to IVF. *Hum Reprod.* 1999;14:2762–9.
119. Zeyneloglu HB, Arici A, Olive DL. Adverse effects of hydrosalpinx on pregnancy rates after in vitro fertilization-embryo transfer. *Fertil Steril.* 1998;70:492–9.
120. Seli E, et al. Removal of hydrosalpinges increases endometrial leukaemia inhibitory factor (LIF) expression at the time of the implantation window. *Hum Reprod.* 2005;20:3012–17. doi: [10.1093/humrep/dei188](https://doi.org/10.1093/humrep/dei188).
121. Bildirici I, Yuksel O, Ensari A, Yarali H, Gurgan T. A prospective evaluation of the effect of salpingectomy on endometrial receptivity in cases of women with communicating hydrosalpinges. *Hum Reprod.* 2001;16:2422–6.
122. Melo P, et al. Surgical treatment for tubal disease in women due to undergo in vitro fertilisation. *Cochrane Database Syst Rev.* 2020;10:CD002125. doi: [10.1002/14651858.CD002125.pub4](https://doi.org/10.1002/14651858.CD002125.pub4).
123. Bellver J, et al. The role of thrombophilia and thyroid autoimmunity in unexplained infertility, implantation failure and recurrent spontaneous abortion. *Hum Reprod.* 2008;23:278–84. doi: [10.1093/humrep/dem383](https://doi.org/10.1093/humrep/dem383).
124. Qublan HS, et al. Acquired and inherited thrombophilia: Implication in recurrent IVF and embryo transfer failure. *Hum Reprod.* 2006;21:2694–8. doi: [10.1093/humrep/del203](https://doi.org/10.1093/humrep/del203).

125. Safdarian L, et al. Recurrent IVF failure and hereditary thrombophilia. *Iran J Reprod Med.* 2014;12:467–70.
126. Steinvil A, et al. Association of common thrombophilias and antiphospholipid antibodies with success rate of in vitro fertilisation. *Thromb Haemost.* 2012;108:1192–7. doi: [10.1160/TH12-06-0381](https://doi.org/10.1160/TH12-06-0381).
127. Stern C, Chamley L, Norris H, Hale L, Baker HW. A randomized, double-blind, placebo-controlled trial of heparin and aspirin for women with in vitro fertilization implantation failure and antiphospholipid or antinuclear antibodies. *Fertil Steril.* 2003;80:376–83. doi: [10.1016/s0015-0282\(03\)00610-1](https://doi.org/10.1016/s0015-0282(03)00610-1).
128. Qublan H, et al. Low-molecular-weight heparin in the treatment of recurrent IVF-ET failure and thrombophilia: A prospective randomized placebo-controlled trial. *Hum Fertil (Camb).* 2008;11:246–53. doi: [10.1080/14647270801995431](https://doi.org/10.1080/14647270801995431).
129. Urman B, et al. Luteal phase empirical low molecular weight heparin administration in patients with failed ICSI embryo transfer cycles: A randomized open-labeled pilot trial. *Hum Reprod.* 2009;24:1640–7. doi: [10.1093/humrep/dep086](https://doi.org/10.1093/humrep/dep086).
130. De Vos A, Van Steirteghem A. Zona hardening, zona drilling and assisted hatching: New achievements in assisted reproduction. *Cells Tissues Organs.* 2000;166:220–7.
131. Fehilly CB, Cohen J, Simons RF, Fishel SB, Edwards RG. Cryopreservation of cleaving embryos and expanded blastocysts in the human: A comparative study. *Fertil Steril.* 1985;44:638–44.
132. Cohen J, Inge KL, Suzman M, Wiker SR, Wright G. Video cinematography of fresh and cryopreserved embryos: A retrospective analysis of embryonic morphology and implantation. *Fertil Steril.* 1989;51:820–7.
133. Malter HE, Cohen J. Blastocyst formation and hatching in vitro following zona drilling of mouse and human embryos. *Gamete Res.* 1989;24:67–80. doi: [10.1002/mrd.1120240110](https://doi.org/10.1002/mrd.1120240110).
134. Cohen J. Assisted hatching of human embryos. *J In Vitro Fert Embryo Transfer.* 1991;8:179–90.
135. Cohen J, Alikani M, Trowbridge J, Rosenwaks Z. Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Hum Reprod.* 1992;7:685–91.
136. Khalifa EA, Tucker MJ, Hunt P. Cruciate thinning of the zona pellucida for more successful enhancement of blastocyst hatching in the mouse. *Hum Reprod.* 1992;7:532–6.
137. Fong CY, et al. Blastocyst transfer after enzymatic treatment of the zona pellucida: Improving in-vitro fertilization and understanding implantation. *Hum Reprod.* 1998;13:2926–32.
138. Valojerdi MR, Eftekhari-Yazdi P, Karimian L, Ashtiani SK. Effect of laser zona pellucida opening on clinical outcome of assisted reproduction technology in patients with advanced female age, recurrent implantation failure, or frozen-thawed embryos. *Fertil Steril.* 2008;90:84–91. doi: [10.1016/j.fertnstert.2007.06.005](https://doi.org/10.1016/j.fertnstert.2007.06.005).
139. Laufer N, et al. The efficacy and safety of zona pellucida drilling by a 193-nm excimer laser. *Fertil Steril.* 1993;59:889–95.
140. Nakayama T, et al. Clinical application of a new assisted hatching method using a piezo-micromanipulator for morphologically low-quality embryos in poor-prognosis infertile patients. *Fertil Steril.* 1999;71:1014–8.
141. Hershlag A, Feng HL. Effect of prefreeze assisted hatching on post-thaw survival of mouse embryos. *Fertil Steril.* 2005;84:1752–4. doi: [10.1016/j.fertnstert.2005.05.065](https://doi.org/10.1016/j.fertnstert.2005.05.065).
142. Antinori S, et al. Zona opening of human embryos using a non-contact UV laser for assisted hatching in patients with poor prognosis of pregnancy. *Hum Reprod.* 1996;11:2488–92.
143. Chao KH, et al. Assisted hatching increases the implantation and pregnancy rate of in vitro fertilization (IVF)-embryo transfer (ET), but not that of IVF-tubal ET in patients with repeated IVF failures. *Fertil Steril.* 1997;67:904–8.
144. Stein A, et al. Assisted hatching by partial zona dissection of human pre-embryos in patients with recurrent implantation failure after in vitro fertilization. *Fertil Steril.* 1995;63:838–41.
145. Petersen CG, et al. Implantation failures: Success of assisted hatching with quarter-laser zona thinning. *Reprod Biomed Online.* 2005;10:224–9.
146. Magli MC, et al. Rescue of implantation potential in embryos with poor prognosis by assisted zona hatching. *Hum Reprod.* 1998;13:1331–5.
147. Martins WP, Rocha IA, Ferriani RA, Nastri CO. Assisted hatching of human embryos: A systematic review and meta-analysis of randomized controlled trials. *Hum Reprod Update.* 2011;17:438–53. doi: [10.1093/humupd/dmr012](https://doi.org/10.1093/humupd/dmr012).
148. Das S, Blake D, Farquhar C, Seif MM. Assisted hatching on assisted conception (IVF and ICSI). *Cochrane Data Syst Rev.* 2009;CD001894. doi: [10.1002/14651858.CD001894.pub4](https://doi.org/10.1002/14651858.CD001894.pub4).
149. Hsieh YY, et al. Laser-assisted hatching of embryos is better than the chemical method for enhancing the pregnancy rate in women with advanced age. *Fertil Steril.* 2002;78:179–82.
150. Primi MP, et al. A European multicentre prospective randomized study to assess the use of assisted hatching with a diode laser and the benefit of an immunosuppressive/antibiotic treatment in different patient populations. *Hum Reprod.* 2004;19:2325–33. doi: [10.1093/humrep/deh430](https://doi.org/10.1093/humrep/deh430).
151. Balaban B, et al. A comparison of four different techniques of assisted hatching. *Hum Reprod.* 2002;17:1239–43.
152. Blake DA, Forsberg AS, Johansson BR, Wiklund M. Laser zona pellucida thinning—an alternative approach to assisted hatching. *Hum Reprod.* 2001;16:1959–64.
153. Lensen S, et al. A randomized trial of endometrial scratching before in vitro fertilization. *N Engl J Med.* 2019;380:325–34. doi: [10.1056/NEJMoa1808737](https://doi.org/10.1056/NEJMoa1808737).
154. Finn CA, Pope M. Vascular and cellular changes in the decidualized endometrium of the ovariectomized mouse following cessation of hormone treatment: A possible model for menstruation. *J Endocrinol.* 1984;100:295–300.
155. Gnainsky Y, et al. Local injury of the endometrium induces an inflammatory response that promotes successful implantation. *Fertil Steril.* 2010;94:2030–6. doi: [10.1016/j.fertnstert.2010.02.022](https://doi.org/10.1016/j.fertnstert.2010.02.022).
156. Barash A, et al. Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing in vitro fertilization. *Fertil Steril.* 2003;79:1317–22.
157. Karimzadeh MA, Ayazi Rozbahani M, Tabibnejad N. Endometrial local injury improves the pregnancy rate among recurrent implantation failure patients undergoing in vitro fertilisation/intra cytoplasmic sperm injection: A randomised clinical trial. *Aus N Z J Obstet Gynaecol.* 2009;49, 677–680. doi: [10.1111/j.1479-828X.2009.01076.x](https://doi.org/10.1111/j.1479-828X.2009.01076.x).
158. Narvekar SA, et al. Does local endometrial injury in the non-transfer cycle improve the IVF-ET outcome in the subsequent cycle in patients with previous unsuccessful IVF? A randomized controlled pilot study. *J Hum Reprod Sci.* 2010;3:15–19. doi: [10.4103/0974-1208.63116](https://doi.org/10.4103/0974-1208.63116).
159. Nastri CO, Ferriani RA, Raine-Fenning N, Martins WP. Endometrial scratching performed in the non-transfer cycle and outcome of assisted reproduction: A randomized controlled trial. *Ultrasound Obstet Gynecol.* 2013;42:375–82. doi: [10.1002/uog.12539](https://doi.org/10.1002/uog.12539).
160. Yeung TW, et al. The effect of endometrial injury on ongoing pregnancy rate in unselected subfertile women undergoing in vitro fertilization: A randomized controlled trial. *Hum Reprod.* 2014;29:2474–81. doi: [10.1093/humrep/deu213](https://doi.org/10.1093/humrep/deu213).
161. Gibreal A, Badawy A, El-Refai W, El-Adawi N. Endometrial scratching to improve pregnancy rate in couples with unexplained subfertility: A randomized controlled trial. *J Obstet Gynaecol Res.* 2013;39:680–4. doi: [10.1111/j.1447-0756.2012.02016.x](https://doi.org/10.1111/j.1447-0756.2012.02016.x).
162. Baum M, et al. Does local injury to the endometrium before IVF cycle really affect treatment outcome? Results of a randomized placebo controlled trial. *Gynecol Endocrinol.* 2012;28:933–6. doi: [10.3109/09513590.2011.650750](https://doi.org/10.3109/09513590.2011.650750).

163. Dain L, et al. Effect of local endometrial injury on pregnancy outcomes in ovum donation cycles. *Fertil Steril.* 2014;102:1048–54. doi: [10.1016/j.fertnstert.2014.06.044](https://doi.org/10.1016/j.fertnstert.2014.06.044).
164. Melnick AP, et al. Autologous endometrial coculture biopsy: Is timing everything? *Fertil Steril.* 2015;104:104–9.e101. doi: [10.1016/j.fertnstert.2015.04.026](https://doi.org/10.1016/j.fertnstert.2015.04.026).
165. Werner MD, et al. Endometrial disruption does not improve implantation in patients who have failed the transfer of euploid blastocysts. *J Assist Reprod Genet.* 2015;32:557–62. doi: [10.1007/s10815-015-0435-0](https://doi.org/10.1007/s10815-015-0435-0).
166. van Hoogenhuijze NE, et al. Endometrial scratching in women with one failed IVF/ICSI cycle—outcomes of a randomised controlled trial (SCRaTCH). *Hum Reprod.* 2021;36:87–98. doi: [10.1093/humrep/deaa268](https://doi.org/10.1093/humrep/deaa268).
167. Simon C, Bellver J. Scratching beneath ‘the scratching case’: Systematic reviews and meta-analyses, the back door for evidence-based medicine. *Hum Reprod.* 2014;29:1618–21. doi: [10.1093/humrep/deu126](https://doi.org/10.1093/humrep/deu126).
168. Yeung WS, Lau EY, Chan ST, Ho PC. Coculture with homologous oviductal cells improved the implantation of human embryos—a prospective randomized control trial. *J Assist Reprod Genet.* 1996;13:762–7.
169. Nieto FS, Watkins WB, Lopata A, Baker HW, Edgar DH. The effects of coculture with autologous cryopreserved endometrial cells on human in vitro fertilization and early embryo morphology: A randomized study. *J Assist Reprod Genet.* 1996;13:386–9.
170. Plachot M, et al. Granulosa cells improve human embryo development in vitro. *Hum Reprod.* 1993;8:2133–40.
171. Quinn P, Margalit R. Beneficial effects of coculture with cumulus cells on blastocyst formation in a prospective trial with supernumerary human embryos. *J Assist Reprod Genet.* 1996;13:9–14.
172. Sakkas D, Jaquenoud N, Leppens G, Campana A. Comparison of results after in vitro fertilized human embryos are cultured in routine medium and in coculture on vero cells: A randomized study. *Fertil Steril.* 1994;61:521–5.
173. Wiemer KE, et al. Beneficial aspects of co-culture with assisted hatching when applied to multiple-failure in-vitro fertilization patients. *Hum Reprod.* 1996;11:2429–33.
174. Wiemer KE, et al. Embryonic morphology and rate of implantation of human embryos following co-culture on bovine oviductal epithelial cells. *Hum Reprod.* 1993;8:97–101.
175. Hu Y, Maxson WS, Hoffman DI, Eager S, Dupre J. Coculture of human embryos with buffalo rat liver cells for women with decreased prognosis in in vitro fertilization. *Am J Obstet Gynecol.* 1997;177:358–362; discussion 362–3.
176. Bongso A, et al. Improved quality of human embryos when co-cultured with human ampullary cells. *Hum Reprod.* 1989;4:706–13.
177. Barmat LI, et al. Human preembryo development on autologous endometrial coculture versus conventional medium. *Fertil Steril.* 1998;70:1109–13.
178. Barmat LI, et al. Autologous endometrial co-culture in patients with repeated failures of implantation after in vitro fertilization-embryo transfer. *J Assist Reprod Genet.* 1999;16:121–7.
179. Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: Key pieces of the development puzzle. *Science.* 1994;266:1508–18.
180. Liu HC, et al. Human endometrial stromal cells improve embryo quality by enhancing the expression of insulin-like growth factors and their receptors in cocultured human preimplantation embryos. *Fertil Steril.* 1999;71:361–7.
181. Tazuke SI, Giudice LC. Growth factors and cytokines in endometrium, Embryonic development, and maternal: Embryonic interactions. *Semin Reprod Endocrinol.* 1996;14:231–45. doi: [10.1055/s-2007-1016333](https://doi.org/10.1055/s-2007-1016333).
182. Jayot S, et al. Coculture of embryos on homologous endometrial cells in patients with repeated failures of implantation. *Fertil Steril.* 1995;63:109–14.
183. Simon C, et al. Coculture of human embryos with autologous human endometrial epithelial cells in patients with implantation failure. *J Clin Endocrinol Metabol.* 1999;84:2638–46. doi: [10.1210/jcem.84.8.5873](https://doi.org/10.1210/jcem.84.8.5873).
184. Eyheremendy V, et al. Beneficial effect of autologous endometrial cell coculture in patients with repeated implantation failure. *Fertil Steril.* 2010;93:769–73. doi: [10.1016/j.fertnstert.2008.10.060](https://doi.org/10.1016/j.fertnstert.2008.10.060).
185. Spandorfer SD, et al. Importance of the biopsy date in autologous endometrial cocultures for patients with multiple implantation failures. *Fertil Steril.* 2002;77:1209–13.
186. Kattal N, Cohen J, Barmat LI. Role of coculture in human in vitro fertilization: A meta-analysis. *Fertil Steril.* 2008;90:1069–76. doi: [10.1016/j.fertnstert.2007.07.1349](https://doi.org/10.1016/j.fertnstert.2007.07.1349).

ULTRASONOGRAPHY IN ASSISTED REPRODUCTION

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Introduction

Can we imagine ART today without imaging? Ultrasound has become the most widely used and important tool in the diagnosis and treatment of infertility. When a patient presents with infertility, ultrasound evaluation is the key part of the exam performed to evaluate the ovaries, uterus, and fallopian tubes. The saline sonogram is used most commonly as an evaluation of the uterine cavity before ART and can identify both congenital and acquired anomalies as well as tubal patency and the presence of hydrosalpinges. This initial ultrasound exam of the ovaries includes an antral follicle count (AFC) for ovarian reserve, diagnosis of polycystic appearing ovaries, endometriosis, and other adnexal pathologies. When ART treatment begins, ultrasound is used for monitoring of follicular development and endometrial response and is critical in the success of the cycle. Ultrasound guided procedures for oocyte retrieval and embryo transfer (ET) are standard practice and ultrasound guidance is helpful in the treatment of Asherman's syndrome and congenital anomalies such as a large septate uterus. Of course, the goal of ART is a singleton viable pregnancy, and early ultrasound monitoring can evaluate the location and viability of the pregnancy and the existence of multiples or vanishing twins.

The quality of the new ultrasound machines and the use of 3-dimensional (3D) ultrasound allow better imaging as well as more accurate diagnosis of pathology and pre-operative preparation. 3D ultrasound has become a gold standard for the diagnosis of uterine anomalies and may assist in more accurate follicular monitoring measurements. Doppler modalities of ultrasound allow identification of the direction and magnitude of blood flow and calculation of velocity useful in separating pathology from normal.

This chapter is aimed to review how 2D and 3D ultrasound are used to maximize ART outcome, concentrating more on the use of 3D. When we see better, we do ART better.

Ultrasound and the ovary

The ovaries are composed of germ cells, stromal cells, and epithelium. The ovaries are visualized with a variety of growing follicles. During puberty, the ovaries enlarge as the follicles grow. Changes in the sizes of the follicles are due to secretion of FSH and LH. The ovaries contain several subtypes of follicles: the primordial follicles, primary follicles, secondary follicles, preantral follicles, and antral follicles (>2 mm diameter). The antral follicles are visible as small cysts and are the smallest follicles that are visible on ultrasound. Follicles grow in two stages, the gonadotropin independent and gonadotropin dependent stages, and the recruitment occurs over three months. Antral follicles are gonadotropin dependent and best evaluated on cycle day 2 or 3. In the early follicular phase, the antral follicles (AFs) that measure from 2 to 10 mm and represent the pool of follicles that may be recruited

in the follicular phase for ovulation. In a natural cycle, the dominant follicles reach a diameter of 17–24 mm prior to ovulation. Ovarian blood flow in an ovulatory cycle is constant up to the point of ovulation. Ovarian flow velocity tends to increase at and immediately after ovulation [1, 2]. After ovulation, a corpus luteum (CL) is frequently seen during the secretory phase of the cycle. It is well vascularized and may have the appearance of a "ring of fire" from the vascularity as seen by power Doppler [3].

A normal CL has a variety of sonographic appearances. Most commonly, the CL appears as a round anechoic cystic mass with a homogeneous, thick, moderately echogenic wall. The cyst is highly vascular with low impedance blood flow and a low-resistance arterial waveform. Haemorrhage into a CL can create a sonographic pattern of internal echoes (Figure 58.1). CL cyst and haemorrhagic cyst have layers that jingle, and rupture of the cyst can result in haemorrhage or clot surrounding the ovary or within the peritoneal cavity.

Ovarian reserve

Ovarian reserve can be indirectly measured by counting the number of antral follicles (measuring 2–10 mm) in each ovary. Age, previous surgery, chemotherapy, and genetics can all affect ovarian reserve, as women are born with a fixed number of oocytes and oocyte loss can be accelerated by the preceding. The peak number of five million primordial follicles occurs prior to birth at about 20 weeks' gestation, and the decrease in the number of oocytes is the result of atresia and is associated with a decrease in the oocyte quality [4]. At birth there are about one to two million oocytes, and at puberty approximately 250,000 oocytes. The exponential loss of follicles accelerates at 37–38 years (only about 25,000), and leads to full depletion of the oocytes at menopause at the average age of 51 [5]. About 400 follicles will achieve pre-ovulatory maturation and ovulate between menarche and menopause. Retrieval of 10–100 of oocytes with multiple IVF cycles does not seem to significantly affect this age-related follicular loss.

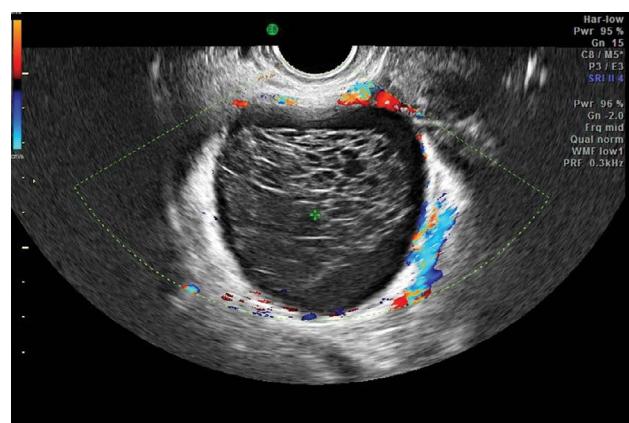


FIGURE 58.1 Haemorrhagic cyst with no flow into the cyst.

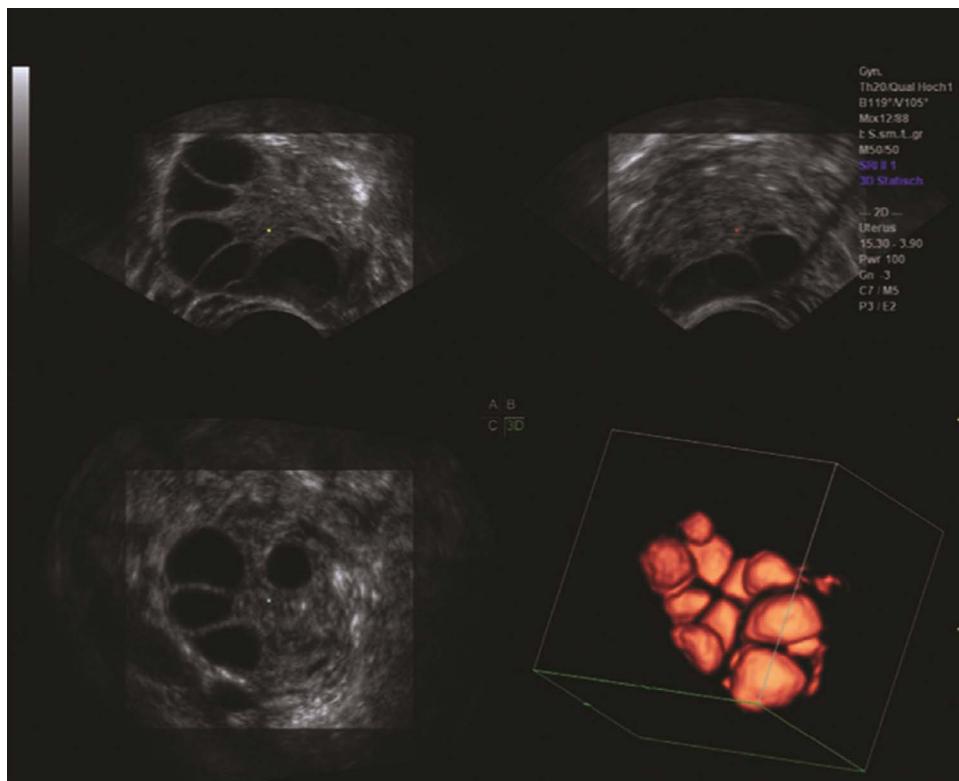


FIGURE 58.2 3D antral follicle count using inverse mode.

Blood tests for estimating ovarian reserve include day 3 FSH and oestradiol (E2) levels as well as anti-Müllerian hormone (AMH) and Inhibin B levels [6–8]. 3D ultrasound for AFC and ovarian volumes are more reproducible and accurate than 2D [9]. However, this method is not universally used, as 3D technology is not freely available for all reproductive endocrinologists and increases the cost. Figure 58.2 shows a 3D AFC and volumes. Both AFC and AMH are the superior methods for predicting ovarian reserve and to predict the response to treatment [10]. These methods are equal in predicting the number of oocytes retrieved in an IVF stimulated cycle [11]. Unlike biochemical parameters, ultrasound is the only method so far that allows a direct assessment of each ovary separately. In addition, the size of the antral follicles on day 2 is important as the larger follicles (>6 mm) are more likely to rapidly grow and lead to atretic or poor-quality oocytes. Determination of the pre-treatment AFC and the number of stimulation selectable follicles help physicians determine patients who are likely to respond poorly during IVF treatment. Those couples can be counselled regarding cycle cancellation and lower chance of success. On the other end of the spectrum, identification of those with a high risk of hyperstimulation allows adjustment of the dose and use of protocols to reduce the corresponding risks and avoid cycle cancellation. Several meta-analyses showed that the AFC correlated with oocyte number and hypo- and hyper-response better than other parameters such as FSH or age [12, 13]. Direct comparison of AFC to AMH levels has shown equivalent predictive values for ovarian response. However, AFC and AMH correlate less well with pregnancy outcomes, which is the more important outcome for the patient than oocyte number. The validity of AFC for ovarian reserve comes from studies demonstrating a direct correlation with the number of non-growing follicles viewed on histological sections [14].

Other ultrasound parameters such as ovarian volume, vascularization, and perfusion had no significant value in predicting poor ovarian response and are inferior to AFC [15]. In women with low AFC, especially at a young age, there is a decrease in quantity but not in quality of the oocytes. Therefore, AFC is highly predictive for the ovarian reserve and strongly associated with the serum AMH level [16]. The estimation of the ovarian response by US is simple and reliable.

The 3D AFC may be a better predictor for poor ovarian response, ovarian hyperstimulation syndrome (OHSS) and the number of oocytes collected, than 2D AFC. Standardization of AFC may improve with the 3D automated identification of the follicles with the post hoc image analysis [17]. Although there are limited studies, the automated imaging can reduce intra- and inter-observer variability and can be reviewed later. Scheffer et al. compared healthy volunteers with proven fertility to patients visiting an infertility clinic [18]. For each patient, 2D or 3D TVS were conducted for AFC (2–10 mm) and inter-observer reliability was calculated. Both techniques were equivalent when only a few follicles were present; however, when higher AFCs occurred, the reproducibility decreased with the 2D technique. In addition to this report, some studies have demonstrated an improvement of inter-observer/intra-observer reliability by the application of 3D methods (in particular by automated systems, such as SONO-AVC) [19]. In addition, they measured the examination time, including time for post-processing of the US scans, and still found less time involved with 3D.

Ovarian cysts—normal and pathology

Ultrasound is the best method for evaluating the ovaries for cysts, and it is a mandatory step in the initial evaluation of the infertile woman. The most common ovarian cysts seen in infertility



FIGURE 58.3 Endometrioma with typical ground glass appearance.



FIGURE 58.4 Dermoid cyst complex with hair and fat.

patients are simple functional cysts, haemorrhagic cysts, endometriomas, and dermoid cysts [20]. Functional follicular or luteal cysts are the most common cystic structures seen in the reproductive age group and they tend to resolve spontaneously within a few months. If they are small (<3 cm) and not hormonally active, they do not need to be treated before ART. However, patients with low ovarian reserve and large simple ovarian cysts may have lower response to stimulation. Ovarian cyst aspiration under ultrasound guidance, with local or IV sedation, immediately prior to ovarian stimulation, has been shown to be beneficial [21]. An endometrioma is also a common finding in the infertile patient and is a sign of the presence of endometriosis in other areas [22]. The typical endometrioma is a unilocular cyst with homogeneous low-level internal echogenicity (ground glass echogenicity) of the cyst fluid (Figure 58.3). An ultrasound diagnosis can avoid surgery in the asymptomatic patient and lead to a decision to move to IVF earlier. During IVF stimulation, one should avoid aspiration of an endometrioma because of an increased risk of infection, compared with aspiration of a simple cyst. Studies show that the presence of an endometrioma is associated with lower response to ovarian stimulation; however, removing the endometrioma prior to stimulation can also affect ovarian response and may significantly diminish ovarian reserve [23–25]. A recent study of 112,475 IVF cycles from published CDC data showed that endometriosis cycles had decreased oocyte yield and higher cancellation rates but no differences in pregnancy or live birth rates compared with male factor patients [25].

Ovarian surgery for endometriosis does not result in improved ART outcome, but, on the contrary, may compromise ovarian reserve [22–26]. Therefore, in the asymptomatic woman, the recommendation is not to intervene prior to IVF. If surgery is performed, more conservative treatment of partial removal and burning of the base may be preferential to a full ovarian cystectomy with laparoscopic stripping of an endometrioma [26]. This recommendation is in line with results from our group [27].

Dermoid cysts or ovarian teratomas are a common ovarian neoplasm in young women of reproductive age and can present as solid hyperechoic heterogeneous masses with a mixed pattern of solid and cystic areas (Figure 58.4). They contain different elements and may contain calcifications, fat, and hair, giving a variable appearance, but commonly the tip of the iceberg sign. They should be removed prior to IVF if they are causing pain or if there is a question of malignancy. Puncture during oocyte retrieval

should be avoided due to high risk of peritonitis. Dermoid cysts should be removed if they are >4 cm as they can rupture or torsion with increased pain during pregnancy [28].

Ultrasound and polycystic ovary (PCO)

Polycystic ovaries detected by transvaginal ultrasonography may be found in approximately 75% of women with a clinical diagnosis of polycystic ovarian syndrome (PCOS) [29–31]. Ultrasound is one of the criteria for the diagnosis of PCOS based on the Rotterdam Consensus conference [32–34]. In addition, polycystic ovaries independently, without the full syndrome, constitute a risk factor for the development of OHSS and the stimulation protocol chosen should reduce the risk. The International Guideline for the Assessment and Management of PCOS and the related translation program aims to provide clinicians with a quality, reliable source of international evidence-based recommendations to guide consistent clinical practice and to empower women with evidence-based information. They endorse the Rotterdam PCOS Diagnostic Criteria in adults. In adolescents, both oligo-anovulation and hyperandrogenism are required, with ultrasound not recommended for diagnosis, due to the high incidence of multifollicular ovaries in this life stage. Ultrasound criteria are refined with advancing technology. Using endovaginal ultrasound transducers with a frequency bandwidth that includes 8 MHz, the threshold for polycystic ovarian morphology (PCOM) on either ovary, is a follicle number per ovary of ≥20 and/or an ovarian volume ≥10 mL on either ovary, ensuring no corpora lutea, cysts, or dominant follicles are present [35] (Figure 58.5).

3D ultrasound, and the use of colour and pulsed Doppler ultrasound showing increased ovarian blood flow, are techniques that further enable the identification of PCO, but are not mandatory for the diagnosis [36]. The preferred stimulation protocol in PCOS patients is the antagonist cycle with GnRH agonist trigger to significantly reduce the risk of OHSS [37].

Ovarian stimulation for IVF—2D and 3D sono AVC

Approximately two-thirds of women develop two follicle waves throughout an interovulatory interval and the remainder exhibit three waves of follicle development. Major waves are those in which a dominant follicle develops; dominant follicles either regress or ovulate. In minor waves, physiologic selection of a dominant follicle is not manifest. Knowledge of waves of antral follicular development has led to the global adoption of novel

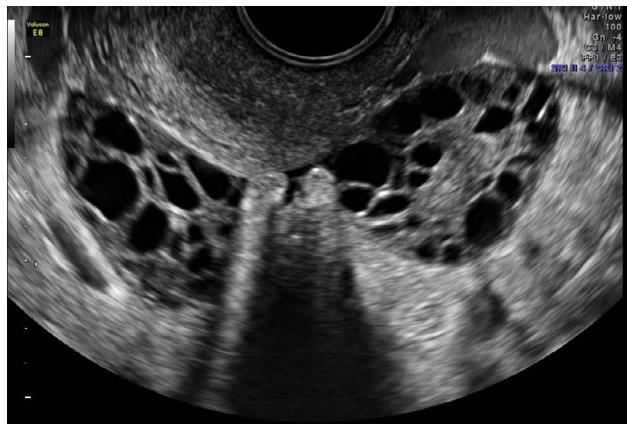


FIGURE 58.5 Polycystic-appearing ovary.

ovarian stimulation strategies in which stimulation can be initiated at various times throughout the cycle [38]. Transvaginal sonography has a vital role in monitoring the follicular growth rate in women receiving ovulation induction medications. Ultrasound monitoring of follicle growth during gonadotropin stimulation was first performed in 1978 [39]. Gonadotropins cause growth of the cohort of follicles at various stages of development and monitoring with serial ultrasound and serum oestradiol is routine with the hope of reducing the risks of OHSS and multiple births. However, data from the Cochrane Collaboration indicate that there is no evidence from randomized trials to support cycle monitoring by ultrasound plus serum oestradiol; it is not more efficacious than cycle monitoring by ultrasound only when measuring outcomes of live birth and pregnancy rates [40]. Follicle size in 2D is best estimated by calculating the mean of the maximum follicular diameter in three planes but is more commonly done in two planes. Follicular growth of 1–3 mm per day

is expected once the dominant follicle(s) measure greater than 12 mm. The aim of the use of gonadotropins for controlled ovarian stimulation during an IVF cycle is to obtain the maximum number of mature and good quality oocytes, as the success improves with numbers. Both nuclear and cytoplasmic maturity are critical and the number of days of stimulation is also a consideration in the formula. Common IVF protocols include the use of either GnRH agonists or antagonists, and in both protocols hCG is administered when three dominant follicles reach a diameter of 17 mm or greater. Over- or under-stimulation can affect the quality of the oocyte. Use of 3D ultrasound for measuring follicle volumes instead of diameters is being studied to see if there is an ideal follicular volume to time the trigger, and whether outcomes can be improved with more precise measuring of the follicles [41].

Sonography-based Automated Volume Calculation (SonoAVC, GE medical systems, Zipf, Austria) is one of the 3D methods developed for follicular monitoring during controlled ovarian stimulation and may increase the reproducibility of the results [42]. First, the multiplanar view is used to ensure the ovary is centrally placed and the render mode is selected to generate a 3D volume of interest box. After SonoAVC is implemented, the individual follicles identified are automatically displayed with a specific colour and shown together with their dimensions and relative sizes. Post-processing is required to manually identify those antral follicles that have been missed in the initial automated analysis and these are then added (Figure 58.6). There have been improvements in this technology, so the false positives and negatives are minimized. The total number of follicles is recorded together with the mean follicular diameter, the volume, and the diameter of each follicle calculated using the relaxed sphere technique [19, 43]. The volume calculation is based on a voxel count defined by the axes x, y, and z of the follicles [44]. The application of SonoAVC for IVF was first described by Raine-Fenning et al. in 2007 [43]. Deutch et al. [45] verified the SonoAVC technique using an ultrasound phantom, showed < 0.02 mL error comparing the

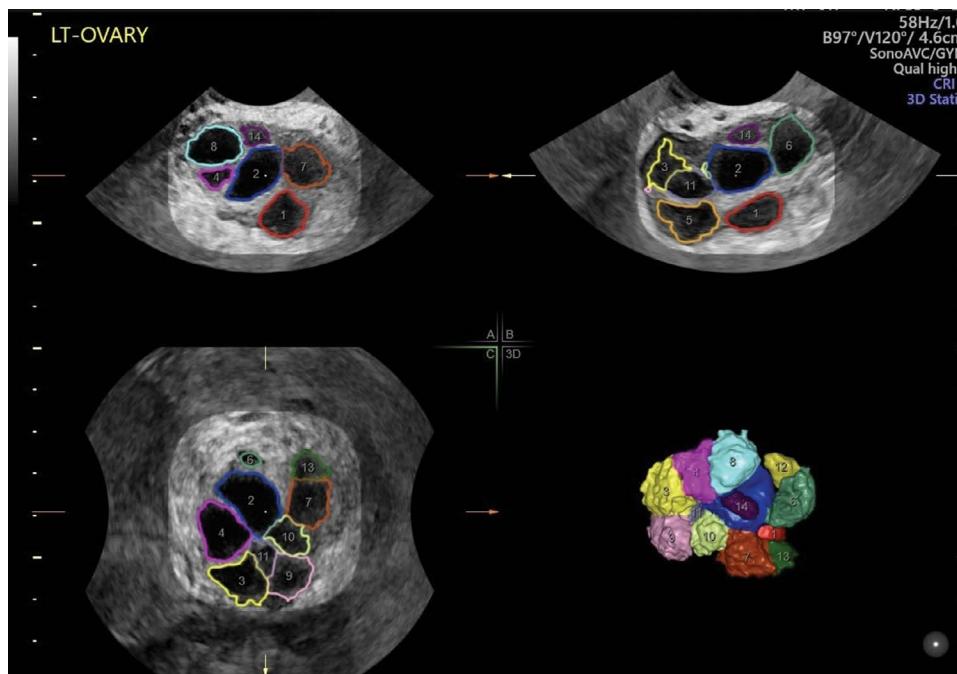


FIGURE 58.6 Automated follicular monitoring with rendered view.

spheres of known volume with a hyperechoic matrix. There is a correlation between the number and size of follicles in stimulated ovaries with SonoAVC and true follicle volume showing the accuracy system for the stimulated ovary. There is also a relationship between the follicular volume calculation and final oocyte maturation and likelihood of collecting mature eggs [46]. However, although SonoAVC leads to standardization of follicle measurements, no studies have shown differences in IVF outcomes using this technique [47]. Rodriguez-Fuentes et al. in their study found a correlation between follicular volume (determined by SonoAVC), day of hCG administration, and retrieval of mature oocytes and follicular volume rather than diameter correlated best with the maturity of the oocytes [48]. They postulated that there is a higher likelihood of obtaining mature oocytes when the follicular volume is ≥ 0.6 mL. However, Raine-Fenning et al. did not find differences in results, in a randomized study [46]. In this study, they used diameter rather than volume-based criteria for determining the timing of hCG injection. Even with similar results, the advantages of SonoAVC may be an ultrasound exam time decrease as the ovarian volumes are saved permitting less discomfort for the patients. Rodriguez-Fuentes found a reduced time savings of four minutes per case after including the post-processing time. Their study has shown that SonoAVC provides more accurate results from those of 2D ultrasound imaging when the size of the follicle is larger [48]. A comprehensive review of SonoAVC is provided by Vandekerckhove et al. reviewing multiple studies and demonstrating the time-saving feature of the SonoAVC studies [49].

Ultrasound of the uterus endometrial thickness and ART monitoring

Ultrasound assessment of the endometrium and the myometrium of the uterus is important in maximizing implantation, both in natural pregnancies and in IVF. Endometrial thickness and pattern varies throughout the menstrual cycle and is the parameter reviewed in most studies [50]. The endometrium is thin immediately after menstruation (2–5 mm), thickens during the proliferative phase, is trilaminar before ovulation, and is thick and echogenic in the secretory phase of the cycle. 3D US has allowed the estimation of the endometrial volume through computer-aided software, VOCAL (Virtual Organ Computer aided Analysis). VOCAL allows the operator to repeatedly outline the endometrium on any plane of the uterus (sagittal, transverse, or coronal), as the uterus is rotated along a stable axis over 180°. In a study by Raine-Fenning et al., the endometrial volume showed remarkable correlation with the 2D US assessment of the endometrial thickness ($R^2 = 0.767$; $P < 0.001$) [51].

Some studies tried to determine certain thresholds for endometrial volume required to achieve a pregnancy, similar to thresholds described for endometrial thickness. Some of these reported significantly reduced pregnancy rates with endometrial volumes of less than 2 mL [52, 53] while others reported no pregnancies in women with an endometrial volume of less than 1 mL [50], or with a volume of more than 8 mL [53]. Due to the conflicting results, and highly heterogeneous data, the data so far do not support the notion that endometrial volume measurement is superior to the measurement of endometrial thickness in predicting the outcome of ART. Transvaginal ultrasound parameters of the endometrium have long been considered implantation markers in IVF, and abnormalities in the uterus explain many causes

for recurrent implantation failure [54–56]. A small amount of endometrial fluid may be seen at the end of stimulation in the middle of the cavity. This is thought to be mucus and can be seen and frequently disappears. However, significant endometrial fluid at the time of ET, usually visible with hydrosalpinges, is associated with poor prognosis, and freezing all the embryos should be considered.

Other assessment of the uterus besides the endometrium includes obtaining the size and position of the uterus and the presence of uterine fibroids or adenomyosis. Assessment of the uterine cavity for fibroids, polyps, adhesions, and Müllerian anomalies is mandatory before ART. Uterine imaging is therefore essential in the diagnosis of infertility. These techniques include conventional hysterosalpingography (HSG), 2D ultrasound, 3D ultrasound, and sonohysterography (SHG). Magnetic resonance imaging (MRI) may be considered only rarely for special cases. This will be discussed in more detail later in the chapter.

Ultrasound examination of the endometrium in a natural or mock cycle supplemented with oestrogen and progesterone provides a non-invasive way to evaluate the endometrial development and receptivity before ET treatment. Synchronization between the endometrial and embryo development is essential for successful implantation.

Sonohysterography (SHG) and the uterus

Sonohysterography (SHG), hysterosonogram (HSN), or saline infusion sonography (SIS) are different names for a minimally invasive office technique used for the evaluation of intrauterine abnormalities. By injecting saline into the uterus, the fluid contrast enhances the visualization of the expanded uterine cavity and filling defects, such as polyps, submucosal fibroids, adhesions, and uterine anomalies, are more easily visualized (Figure 58.7).

Randolph et al. were the first to perform transabdominal ultrasound scan during saline infusion [57]. The main uses for SHG are for abnormal uterine bleeding, screening of the uterine cavity prior to ART, and habitual abortion [58–60]. Contrast media may be also used for injection, such as saline mixed with air or Echovist® (Schering AG, Germany). The American College of Obstetricians and Gynecologists (ACOG) published a bulletin in conjunction with the American Institute of Ultrasound in Medicine (AIUM), the Society for Reproductive Endocrinology



FIGURE 58.7 Saline sonogram of uterine polyp with feeding vessel.

and Infertility (SREI), and with the American College of Radiology (ACR) describing the technique of SHG [61].

Initially, the conventional B-mode transvaginal scan is done to assess the uterus, ovaries, and pouch of Douglas. After gaining consent, the patient is placed into the dorsal lithotomy position. A speculum is inserted into the vagina and the cervix is cleaned with an aseptic solution. A balloon or soft catheter can be used to cannulate the cervix. Once the catheter is in place, the speculum is removed (open sided is easier) and the transvaginal ultrasound (TVUS) probe is inserted to confirm placement. The contrast medium or saline should be injected slowly to decrease bubbles, along with real-time sonographic imaging. Spieldoch et al., in an randomized controlled trial (RCT), showed that cervical placement of the catheter was less painful than intrauterine placement [62]. Intracervical catheter placement resulted in significantly less pain during SHG and also requires half the saline volume to perform. Therefore, intracervical balloon placement should be preferred for SHG [63]. Tubal patency can be assessed if contrast or agitated saline is used to demonstrate flow along the entirety of the tube and spill around the ovary. In most cases with a contrast, fluid can be seen moving from the cornual end distally with spill into the pouch of Douglas. A detailed examination of the uterus is performed by scanning slowly and systematically from cervix to fundus. SHG had been described with gel instillation as well [64, 65].

A 3D image is helpful and can be processed to review any lesions such as fibroids or polyps affecting the cavity. Studies comparing findings on SHG with 2D and 3D ultrasound, HSG, and hysteroscopy show excellent predictive value of SHG and a benefit to the 3D [66–71]. Saline infusion sonography (SIS) is a highly sensitive and specific uterine test in the diagnosis of uterine anomalies and can be used as a screening tool for sub-fertile patients prior to IVF treatment [72]. A recent Cochrane meta-analysis revealed no statistically significant differences between 2D SIS and 3D SIS. Summary sensitivity and summary specificity are higher for 3D SIS, but margins of improvement are limited because 2D SIS is already very accurate [73]. Therefore, both 2D SIS and 3D SIS should be considered screening alternatives to diagnostic hysteroscopy when intracavitary pathology is suspected in sub-fertile women and in those with abnormal uterine bleeding [67].

Acquired uterine abnormalities

Uterine abnormalities are very common both in infertility and abnormal bleeding patients. In a prospective study of 600 infertility patients by Tur-Kaspa et al., 20% were found to have cavitary abnormalities, including arcuate uterus (15%), polyps (13%), submucosal fibroids (3%), and adhesions (1%) [68]. This prospective study compared the incidence of uterine cavity anomalies in patients referred for infertility or abnormal bleeding. More patients in the bleeding group had intracavitary abnormalities such as polyps, fibroids, and adhesions as well as intramural abnormalities, and the infertility group had more congenital uterine anomalies.

In another study by Alborzi et al., SHG (compared with HSG) showed higher specificity and negative predictive values (NPV) for detection of uterine and tubal abnormalities [69]. They reported a sensitivity, specificity, PPV, and NPV of 78.2%, 93.1%, 82.7%, and 91%, respectively, compared to 76.3%, 81.8%, 90.9%, and 59.2%, respectively, for HSG. SHG is very accurate for pathology with less pain and no exposure to radiation, and 3D is desirable to evaluate the entire cavity by using section places to scroll through. There are further advantages of SHG over HSG; the ovaries and other non-cavitory lesions can be seen at the same time.

Multiple studies support the hypothesis that intramural fibroids have a detrimental impact on pregnancy and on implantation rates in IVF, especially when the fibroids are large [74]. This includes caesarean sections, preterm delivery, preterm rupture of membranes, and haemorrhage. The mean gestational age at delivery for women with fibroids larger than 5 cm is 36 weeks, significantly earlier than women with smaller fibroids or no fibroids [75]. Assessment of uterine fibroids has been most commonly achieved using ultrasonography. The large uterus may require abdominal ultrasound as well, but in general TVUS is satisfactory. For intramural and submucosal fibroids, 3D ultrasound, especially in the coronal view, is a way to map the position and distance from the endometrial cavity. The addition of saline infusion can help with the type of surgical approach chosen to remove submucosal fibroids and to subtype the fibroid. SHG is better at showing the cavity involvement and the percentage of the fibroid in the cavity and in the myometrium [76]. The 3D multiplanar display is also useful in some cases for differentiating adnexal lesions close to the uterus from lesions within or originating from the uterus. MRI is superior in selected cases where the fibroids are large and outside of the pelvis leading to shadowing on TVUS [77, 78]. An unpublished study by the authors on the imaging of uterine fibroids reveals that the number of fibroids is underestimated by ultrasound compared to those found at surgery and MRI, even when 3D imaging is used [79]. Use of 3D ultrasound and 3D SHG for determining the position of the fibroids can be visualized in **Figure 58.8**.

Another method that may be helpful to identify fibroids is the use of colour Doppler. Since the fibroid is surrounded by a rich vascular supply, a myoma will usually demonstrate a “ring of fire.” It is important to make the diagnosis of fibroids, and to identify the women that will benefit from a myomectomy. A systematic review confirmed that ART outcomes are decreased in women with submucosal fibroids, and hysteroscopic removal improves the outcome significantly [80]. A later updated meta-analysis confirmed that even non-cavity distorting intramural fibroids were associated with adverse pregnancy outcomes [81]. A Cochrane meta-analysis that reviewed four RCTs concluded that there was limited evidence to determine the role of myomectomy for infertility in women with intramural/submucous fibroids, as only one trial compared myomectomy with no myomectomy [82].

The effect of fibroids on fertility remains controversial. A later updated meta-analysis confirmed that even non-cavity distorting intramural fibroids were associated with adverse pregnancy outcomes [83]. Studies have reported the size thresholds ranging from >2.95 cm to >5 cm at which intramural myomas begin to cause impaired reproductive outcomes [84, 85]. A recent meta-analysis concerning 2D SIS reporting a pooled sensitivity and specificity for diagnosing submucosal fibroids of 82% (95% CI 69%–92%) and 100%, respectively [72]. Well-designed prospective studies comparing 2D US, 2D SIS, 3D US, and 3D SIS found 3D US to be superior to 2D US [73].

Other non-surgical treatments of fibroids, such as uterine artery embolization and magnetic resonance guided focused ultrasound (FUS) procedures are options and pregnancies have been reported [86], although they both carry a higher risk for complications in pregnancy. For uterine artery embolization, MRI is recommended prior to the embolization procedure for best results. The Exablate MRgFUS treatment uses a “sonication” process in which a FUS concentrates a high energy beam on a specific point, raising its temperature and destroying fibroid tissues by coagulation necrosis. Review of the literature on

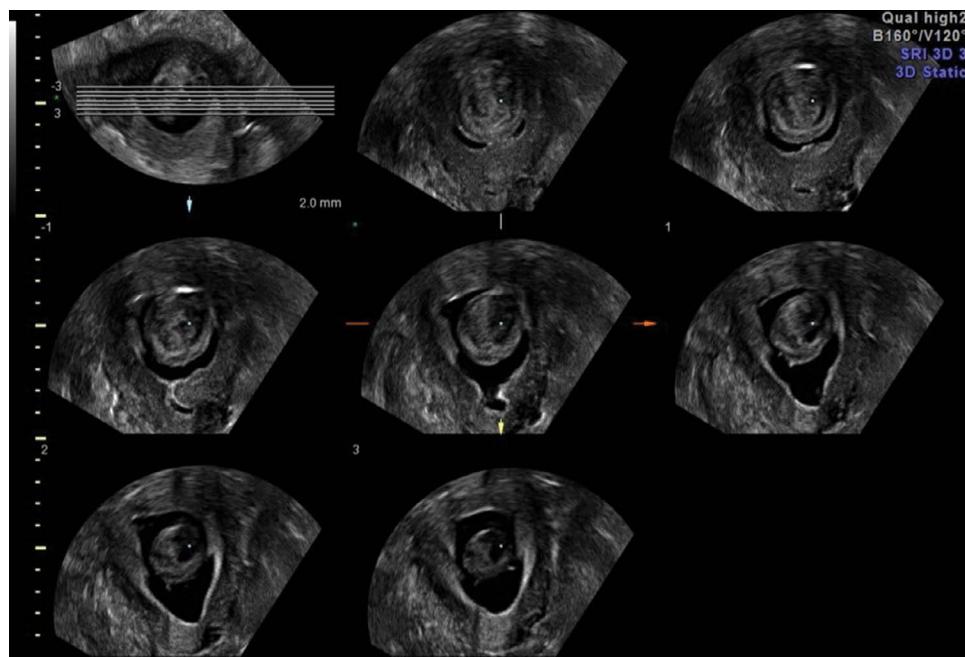


FIGURE 58.8 Saline sonogram of submucosal fibroid multiplanar view.

pregnancy outcome after MRgFUS revealed 88 pregnancy cases. The mean time to conception was eight months after treatment. Of those 88 pregnancies, there were 45 (51%) total deliveries, 67% SVD (spontaneous vaginal deliveries), and 33% C/S (caesarean section). However, 19 of those 88 (22%) women experienced spontaneous abortions. Rabinovici et al. noted that uterine rupture, preterm labour, placental abruption, and abnormal placentation leading to fetal growth restriction were not observed in any of the cases, unlike the UAE procedures [87].

Adenomyosis may have several appearances by ultrasound, making the diagnosis uncertain in some cases. Adenomyosis shows a traditional radiological criteria such as the presence of an enlarged “globular” uterus in the absence of fibroids, asymmetric thickening of the anterior or posterior myometrial wall, heterogeneous poorly circumscribed areas within the myometrium, anechoic myometrial blood-filled lacunae or cysts of varying sizes, increased echo-texture of the endometrium, and sub-endometrial linear striations [88]. On ultrasound, it appears as asymmetry and thickening of the uterine walls with loss of endometrium–myometrium border and hypoechoic nodules in the myometrium. However, adenomyosis may appear hyper-echoic, hypoechoic, or the signal may be mixed. Adenomyosis can enlarge or shrink in throughout a menstrual cycle, depending on the hormonal response. In some cases, adenomyosis forms a nodular myometrial mass which is readily identified by ultrasound which is called an adenomyoma. Adenomyosis can also be a diffuse condition affecting a large segment of the myometrium, with the only ultrasound finding being a subtle uterine enlargement. Sometimes, adenomyosis and uterine fibroids have a remarkably similar appearance with ultrasound, and some women have both conditions. Colour Doppler studies are helpful to distinguish uterine fibroids from adenomyosis, since vascular flow is peripheral with fibroids, and more homogeneously affects adenomyosis lesions. Fibroids must be differentiated from adenomyosis, especially when surgery is considered, since resection of adenomyosis and repair of the defect can be difficult [89, 90]. When assessing

the diagnostic accuracy of 2D US against a confirmed histological diagnosis of adenomyosis, a systematic review and meta-analysis showed that 2D US had a sensitivity of 72% (95% CI 65%–79%), specificity of 81% (95% CI 77%–85%). MRI proved to be slightly superior than 2D US, with a sensitivity of 77% (95% CI 67%–85%), specificity of 89% (95% CI 84%–92%) [91]. In a study by Sharma et al., the parameter of “ill-defined junctional zone on 3D US” ranked as the second-best predictor of adenomyosis after the 2D US marker of central vascularity (sensitivities of 86% and 93%, respectively), and it seems likely that a combination of 2D and 3D US markers is likely to provide the highest diagnostic accuracy for diagnosing adenomyosis and performing objective measurements and mapping of the disease [92] (Figure 58.9). Whether adenomyosis is a cause of infertility is controversial. However, in a meta-analysis of published data, women with adenomyosis



FIGURE 58.9 Adenomyosis with the “venetian blind” shadowing appearance.

had a 28% reduction in the likelihood of a clinical pregnancy with IVF/ICSI [93].

Endometrial polyps are the most common endometrial anomaly and may be found in about 15% of infertile women [68]. TVUS is the imaging of choice, and saline sonography, especially 3D, may be helpful in locating the exact location of the polyp [94–96]. Endometrial polyps appear as ovoid echogenic masses that project into the endometrial lumen without myometrial involvement and are best seen in the follicular phase when the endometrium is the thinnest and less echogenic. Doppler US often shows a feeding vessel in many cases (Figure 58.7); however, a polyp may present as a diffuse thickening of the endometrium which presents a difficulty in the ability to detect polyps [76]. Salim et al. reported that for TVUS, the sensitivity varies between 19% and 96%, specificity of 53%–100%, PPV of 75%–100%, and NPV of 87%–97%, when compared with hysteroscopy with guided biopsy. A recent systematic review and meta-analysis on 2D SIS found the pooled sensitivity and specificity for diagnosing polyps to be 0.82 (95% CI 0.76–0.86) and 0.96 [0.95–0.98], respectively, with positive and negative likelihood ratios of 34.66 (95% CI 8.12–147.92) and 0.22 (95% CI 0.13–0.39), respectively [72]. 3D may improve the results, as Kupesic et al. reported that 3D ultrasound has sensitivity of 100%, specificity of 99%, PPV of 99%, and NPV of 100% in diagnosing endometrial polyps when compared to hysteroscopy with biopsy [97].

Tiras et al. investigated the impact of endometrial polyps on pregnancy rates in 8359 ICSI patients [98]. The study included all fresh ICSI cycles performed in the Anatolia IVF Centre between 2005 and 2009. All patients diagnosed with an endometrial polyp by TVUS before the ICSI cycle underwent hysteroscopic polyp resection. Localization of the polyp (upper, middle, or lower third of the uterine cavity) or polyp size (4–14 mm) did not seem to affect pregnancy rates. They concluded that endometrial polyps less than 1.4 cm found during ovarian stimulation did not affect pregnancy rates, miscarriage rates, and live-birth rates in ICSI cycles, and that patients with an endometrial polyp detected before ICSI treatment and resected by hysteroscopy had similar pregnancy rates compared with patients with no endometrial polyps.

It is controversial whether endometrial polyps contribute to infertility or miscarriages. Some studies show it depends on the location and size of the polyps [98, 99]. A Cochrane meta-analysis suggests that hysteroscopic removal of endometrial polyps suspected on ultrasound in women prior to IUI may improve the clinical pregnancy rate compared to simple diagnostic hysteroscopy [100]. More research is needed to measure the effectiveness of the hysteroscopic treatment of suspected major uterine cavity abnormalities in women with unexplained sub-fertility or prior to IUI, IVF, or ICSI [101]. To date, insufficient data are available to justify the removal of polyps as a routine practice in sub-fertile women. In addition, performing hysteroscopic polypectomy does not compromise reproductive outcomes from subsequent IVF techniques [101]. However, surgery should be performed for sub-mucous fibroids and endometrial polyps when there is recurrent implantation failure or recurrent pregnancy loss. Following surgery, there is no need to wait, as the IVF success is no different the cycle after hysteroscopy compared to waiting a month [98].

Intrauterine adhesions (IUAs) also present as acquired uterine anomalies and are, in most cases, the result of retained products of conception after pregnancy and repeat curettage for incomplete abortions. Myomectomy for intracavitary fibroids and uterine artery embolization are also causes. As mentioned previously,



FIGURE 58.10 Intrauterine adhesions after saline sonogram.

adhesions are not seen that well with basic ultrasonography. There have been reports of MRI appearances in four cases of Asherman's syndrome in which the diagnosis was confirmed by hysteroscopy. However, the full range of MRI appearances in Asherman's syndrome has not been established and there has been only one case reported in the literature [102]. Figure 58.10 shows IUAs using a multiplanar view after SHG. The study by Knopman showed that 3D imaging was very accurate in Asherman's syndrome in a case series of 54 infertile patients with thin endometrial linings [103]. Intrauterine adhesions were diagnosed on 3D ultrasound and HSG in all cases and confirmed by hysteroscopy. They reported 100% sensitivity with 3D ultrasound for correctly grading the extent of IUAs compared to only 66.7% for HSG. HSG over-diagnosed the extent of the Asherman's segment outflow obstruction. For the surgical treatment of Asherman's ultrasound guidance may aid in the hysteroscopic lysis of dense scar tissue and difficult entry into the cervix. Importantly, an obliterated cavity may require multiple hysteroscopic treatments [104, 105]. In the largest study, involving 6680 hysteroscopies with hysteroscopic adhesiolyses in 75 patients, 94.6% functional restoration and 93.3% anatomic resolution, with pregnancy rates ranging from 28.7 to 53.6%, was achieved [106]. At two-month follow-up, the uterine cavity was completely restored in 70 cases, while in four cases a second surgical treatment was necessary.

Congenital uterine anomalies

Müllerian anomalies are congenital defects in the development of the uterus and upper vagina. It has been demonstrated that conventional 2D US imaging is a good screening tool for the detection of congenital uterine anomalies and has a high sensitivity for some anomalies [97]. However, 3D ultrasound, especially the coronal view, is superior to 2D and has been accepted as the first-line test and shown to be as accurate as the MRI in detection of congenital anomalies with difficulty visualizing the vagina [107]. New grading systems have been published based on 3D ultrasound. Precise classification of a uterine anomaly is of clinical importance because the need for surgical intervention and the type of intervention depends on this distinction.

Again, the mid-coronal view is the most important view for congenital anomalies. With 3D ultrasound, a volume of ultrasonographic data is acquired and stored. The stored data can be reformatted and analysed in numerous ways; navigation through the saved volume can demonstrate innumerable arbitrary planes. The optimal time to examine patients for the presence of uterine

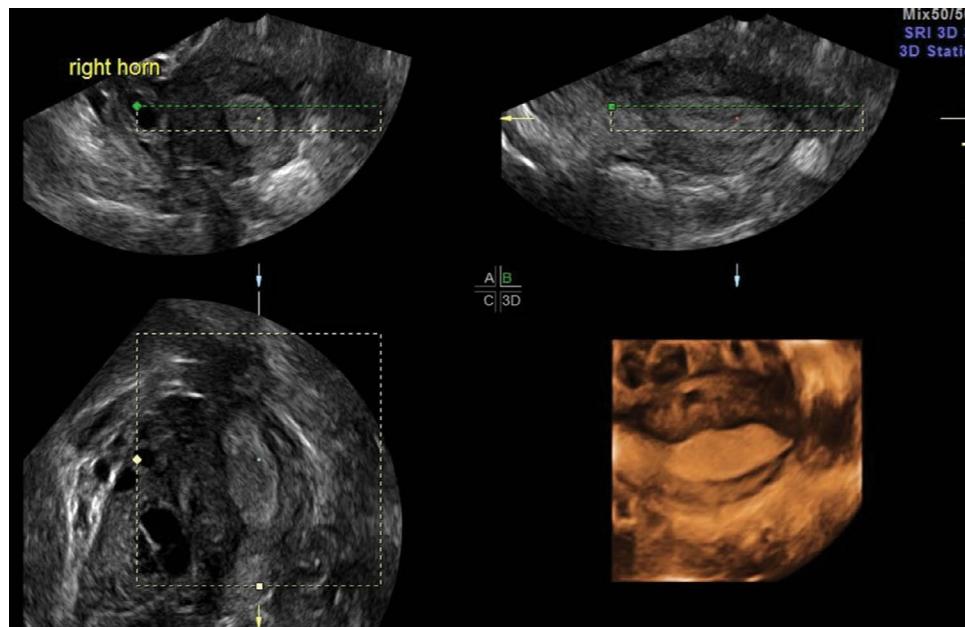


FIGURE 58.11 3D unicornuate uterus manipulation.

anomalies is the luteal phase of the cycle when the endometrium is thick and echogenic and the cavity can be clearly differentiated from the surrounding myometrium. The most important advantage of 3D US over HSG is the ability to visualize both the uterine cavity and myometrium. It provides complete information about the nature and extent of uterine masses and congenital anomalies. A number of studies have shown complete agreement and accuracy between the congenital uterine anomaly seen on 3D ultrasound with that of HSG or hysteroscopy and laparoscopy as the gold standards [108, 109]. Controversy exists over whether an arcuate uterus, defined by an endometrial dip <1 cm, or a septate uterus up to 1.5 cm carries any clinical significance [110]. Figures 58.11–58.13 show the 3D visualization of unicornuate uterus, unicornuate uterus with noncommunicating horn, and a complete septate uterus.

Caesarean section scar niche

Caesarean scar (CS) defect or niche is predominantly an ultrasonographic diagnosis and relates to the presence of a hypoechoic area within the myometrium of the lower segment, reflecting the discontinuation of the myometrium at the site of a previous

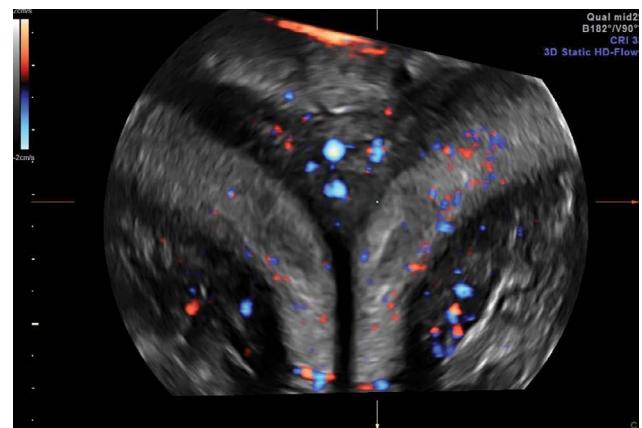


FIGURE 58.13 3D of complete uterine septum.

caesarean section. CS defects may be asymptomatic, present with abnormal bleeding, chronic pelvic pain, secondary infertility where the persistence of blood resulting in chronic inflammation may possibly lead to altered cervical mucus, sperm transport and interfere with embryo implantation, and obstetrical complications including abnormal placentation, scar dehiscence, and uterine rupture [111]. A meta-analysis suggested that patients who had undergone a caesarean section had a 9% significantly lower subsequent pregnancy rate and an 11% significantly lower birth rate [112].

A recent study that compared 2D US with 2D SIS in assessing the myometrial thickness adjacent to the scar defect and the residual myometrial thickness over the scar defect reported excellent intra-/inter-observer reliability (intraclass correlation coefficients of ≥ 0.97) and concluded that 2D SIS in particular is a reliable, reproducible method that can be used in clinical practice to assess CS defects in non-pregnant women [113]. A study assessed the reproducibility of 3D US in the assessment of a CS



FIGURE 58.12 Unicornuate uterus with noncommunicating horn.

defect. The authors found that various niche parameters, including depth, maximal width, and width at niche base, can be measured with a high level of agreement, particularly if measured in the longitudinal plane [114]. In contrast, a subsequent similar study by Glavind et al. reported that US measurements remain subjective with rather wide limits of agreement, even with 3D volumes [115]. Indeed combined 2D/3D SIS may prove to be the most informative examination, as it can clearly depict the extent of the defect not only in terms of depth but also laterality. Three different surgical approaches to managing these defects have been described: hysteroscopic (when the residual myometrium is ≥ 3 mm), laparoscopic, and vaginal. A recent randomized study comparing hysteroscopic resection to observation showed that hysteroscopic management resulted in reduction of median number of post-menstrual spotting from eight days to four days at six months post-surgery [116]. The same group also performed a prospective trial of laparoscopic CS defect resection where a median nine days of spotting resolved to two days and the defect depth reduced from 9.9 mm to 4.2 mm [117]. However, since there was no control group in this study, laparoscopic and hysteroscopic approaches cannot really be compared.

Doppler flow in the endometrium and receptivity in IVF

There is a controversy regarding the value of measuring endometrial thickness (EMT) in predicting pregnancy during IVF treatment. EMT is measured from outside to outside in an anterior-posterior view at the widest point. Patients with a thin endometrium following ovarian stimulation have a significantly lower pregnancy rate but have yielded a high percentage of false positive results [118]. Low dose aspirin, vaginal Sildenafil (Viagra) and pentoxifylline have been used to treat patients with thin endometrium [119, 120]. The underlying assumption is that patients with thin endometrium have suboptimal endometrial blood flow and may have scar tissue, and aspirin or Viagra increase the endometrial blood flow and endometrial development. However, studies do not consistently show improvement. The relationship between the EMT and the live birth rate has been investigated in both fresh and frozen-thawed ET cycles. The results for both are conflicting because some attributed substantial importance to the EMT [121, 122], whereas others suggested that the EMT has no or minimal effect on achieving live birth [123, 124]. With increasing EMT (>14 mm), a high miscarriage rate was reported by Weissman et al. [125]. An excessively thick endometrium may start in a previous cycle, so ovarian stimulation should not be started following menstruation if the EMT is greater than 6 mm. Increased pre-clinical or biochemical miscarriages are also seen when the EMT is 6–8 mm versus 9 mm or greater [126]. These findings correlate well with the recent report of increased pregnancy loss with low endometrial volume on the day of the first pregnancy test 14–18 days after oocyte retrieval [127]. The thinnest reported lining in a successful pregnancy was with an EMT of 4 mm, so this remains controversial [128].

3D ultrasound and power Doppler angiography with the aid of the VOCAL[®] (Virtual Organ Computer-Aided Analysis) can be used to provide a fast means of measuring endometrial parameters, such as endometrial volume and angiography blood flow, to predict endometrial receptivity [129, 130]. It was previously believed that uterine arterial resistance changes in 3D might reflect uterine receptivity [131, 132]. Although pregnancy outcomes tended to be poor in patients with higher mean uterine

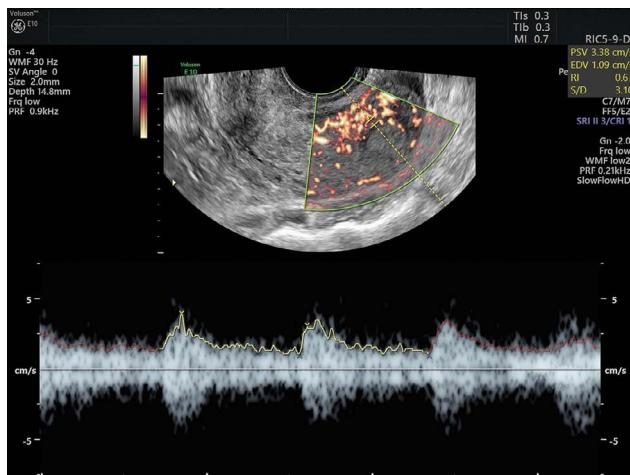


FIGURE 58.14 Slowflow Doppler of the endometrium.

arterial impedance indices, the predictive value of using a specific RI or PI in assessing endometrial receptivity seems to be limited [133].

SlowflowHD is a new technology that can be used to measure low-velocity vascular flow in the small arterioles that feed the endometrium and allows a comparison between the intensity of the colour signals present in the endometrium and sub-endometrium (Figure 58.14). The authors have found that with the use of SlowflowHD, pregnancies and live births were associated with a lower percentage decrease in blood flow from day of progesterone start oestradiol priming to transfer day [134]. There has been much attention paid to the progesterone levels at the time of hCG administration. Several studies have suggested that a premature secretory endometrial pattern is caused by the advanced progesterone rise, and this premature conversion has an adverse effect on pregnancy rates. The reason that no-triple-line endometrial pattern is observed prior to ovulation in some women is not known and cannot be explained by higher progesterone levels. In the study by Ng et al., three patients with calcifications in the uterus and two with fluid in the endometrial cavity on the day of hCG did not conceive [135]. Other poor prognostic factors include fluid in the endometrial cavity or calcifications in the uterus. In these cases, freezing all the embryos until an evaluation of the uterine cavity can be done may be recommended.

In conclusion, although characteristics of the human endometrium, including thickness (volume), morphology, endometrial blood flow, and vascularization, can be readily and non-invasively monitored by US, there still is not a clear and consistent correlation between the patterns and successful implantation. However, the 3D studies show better correlations than 2D.

Ultrasound of the fallopian tubes

Fallopian tube patency

Normal fallopian tubes are usually not visualized by ultrasound. Evaluating tubal patency is a crucial step in the workup for infertility and making a diagnosis of hydrosalpinx is important prior to IVF. HSG has been the standard treatment for assessment of the fallopian tubes but has high false positive rates. When compared to laparoscopy with chromoperturbation, HSG has a false positive rate of 20% and has disadvantages of exposure to radiation

and pain [136]. Tubal occlusion, unilateral or bilateral, is seen in approximately 20% of women with infertility [137].

Richman et al. were the first to describe, in 1984, the use of transabdominal ultrasound for the diagnosis of tubal patency with saline contrast [138]. After transcervical installation of saline, the cul-de-sac was evaluated for the appearance of free fluid. During the preliminary US, the posterior cul-de-sac and pelvis was evaluated for the presence of free fluid. If none is present before injection of fluid and it is present after fluid injection, then it was concluded that at least one tube was patent, but it was not clear which tube. Since the development of this first technique, significant advances have been made in ultrasound technology, including the advent of transvaginal sonography and 3-dimensional volume sonography and other contrast agents [139].

For infertility patients, the advantage of the use of ultrasound is the ability to see the adnexal structures, the uterus for polyps, fibroids, or congenital anomalies, as well as the presence of hydro-salpinges. Additional contrast material or a small amount of air is injected with the fluid with concurrent real-time sonographic imaging in the cornual plane of the adnexae and cul-de-sac to assess tubal patency. Recent studies demonstrate a good correlation with HSG. The ultrasonographic evaluation of tubal patency is referred to as hysterosalpingo-contrast sonography (HyCoSy). HyCoSy can be performed using a negative contrast agent such as saline, or a positive contrast agent such as Echovist 200 [140, 141]. HyCoSy in the US is usually performed by injecting a small amount of saline into the uterus via an intrauterine balloon catheter, as the contrast agent is not FDA approved. During the installation process, a TVUS is performed to assess the tubal flow of contrast material and/or the accumulation of contrast material in the pouch of Douglas. 3D evaluation has been published and a recent systematic review comparing the 3D with 2D shows 3D superiority.

Agitated saline is used in lieu of commercially manufactured contrast material in the US. Agitated saline is produced by placing 19 cc of saline and 1 cc of air in a 20 mL syringe. The syringe is then vigorously shaken and the mixture is injected into the uterus using a balloon catheter [142]. Sonographic criteria for tubal patency were bubbles entering the fallopian tubes without production of a hydrosalpinx or exit of bubbles into the peritoneal cavity. The results showed tubal patency was confirmed in 89% of the tubes.

The disadvantages of HyCoSy include the difficulty at times to follow the passage of contrast through the entire length of the fallopian tube and difficulty to visualize the tube in a single plane. Therefore, 2D HyCoSy requires significant skill on the part of the ultrasonographer [143]. If positive contrast media is used, it can be challenging to differentiate the echogenicity of the contrast material from the surrounding bowel. Therefore, the visualization of true spill from the fimbriated end of the fallopian tube and visualization of the fimbria remains difficult. Tubal pathology such as mucosal folds or salpingitis isthmica nodosa cannot be evaluated using HyCoSy. Still, from the meta-analysis, 3D HyCoSy has been shown to be an accurate test for diagnosing tubal occlusion in women with infertility.

Globally, there is a shift towards the use of office-based diagnostic methods; one such technique is hysterosalpingo-foam sonography (HyFoSy). This is an alternative imaging technique that lacks ionizing radiation and iodinated contrast medium exposure. This technique uses a more stable echogenic medium ExEm-foam that was FDA approved in 2019. A recent multicentre randomized controlled trial compared HyCoSy and HSG. During HyFoSy 5–10 cc of echogenic foam was infused in the



FIGURE 58.15 HyFoSy showing bilateral tubal patency.

uterine cavity through a small cervical balloon-less GIS catheter (IQ Medical Ventures BV, Rotterdam, The Netherlands). The foam was created by rigorously mixing 5 cc ExEm-gel (IQ Medical Ventures BV, Rotterdam, The Netherlands) with 5 cc sterile purified water. The created foam was slowly infused into the uterine cavity during 2-dimensional transvaginal sonography, and subsequently into the fallopian tubes to assess patency ([Figure 58.15](#)). This study showed that relying on either HyFoSy or HSG in infertile women produced similar tubal pathology findings and lead to similar pregnancy outcomes, while HyFoSy was associated with significantly less pain. The authors stated that the use of 3-dimensional or Doppler imaging might increase the accuracy of HyFoSy, make HyFoSy less operator dependent and possibly less time-consuming [[144](#)]. The technique can be combined with saline sonogram for cavity evaluation and foam contrast is easily visualized through the fallopian tubes and into the peritoneal cavity.

Doppler and 3D ultrasound

3D HyCoSy with colour power Doppler has been shown to increase the ability to depict true tubal patency by free spillage of contrast material from the fimbriated end of the fallopian tube. In addition, it better differentiates free fluid of echogenic contrast from the bowel. One study demonstrated that free spill of contrast material was seen 91% of the time with 3D HyCoSy and only 46% of the time with 2D HyCoSy [145]. In addition, 3D HyCoSy with colour power Doppler seems to be accurate, as it was found to agree with laparoscopy with chromoperturbation 99% of the time. Blood flow and Doppler are additional modalities that can be employed in conjunction with HyCoSy [146]. The use of colour Doppler with 2D HyCoSy has also been shown to increase the ability to diagnose true tubal occlusion and help differentiate between the contrast material that is spilling out of the tube and the surrounding bowel.

For 3D, ultrasound should be used to sweep the region of interest along the entire tubal length. As a result, 3D HyCoSy can visualize the volume of the tube and is less operator dependent and easier to perform. 3D colour power Doppler can also be used to depict the flow of contrast material through the entire length of the fallopian tube so it has clear advantages over the use of HyCoSy alone. It has been shown that visualization of distal tubal spill occurs twice as often when 3D colour power Doppler is employed [147]. Since the procedure relies on the technical ability of the clinician performing the procedure, it is still not routinely performed.

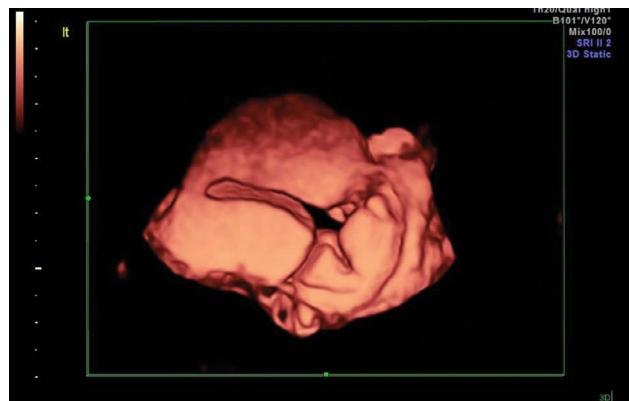


FIGURE 58.16 3D rendered view of hydrosalpinx.

Hydrosalpinges and ART outcome

Hydrosalpinges are common causes of infertility, decreasing IVF pregnancy rates (PR) by 50% [148]. Studies have shown embryo-toxic fluid in the cavity and prophylactic salpingectomy, or tubal ligation, is recommended to improve PRs. Studies show that the hydrosalpinx fluid may affect endometrial development and contraction [149]. If a hydrosalpinx appears during stimulation, ultrasound-guided aspiration of hydrosalpinges at oocyte collection can be an option. A randomized, blinded study showed that 30% of the fallopian tubes in the aspiration group re-accumulated by 14 days after the aspiration [150]. This study implies that the window of opportunity may be present at oocyte aspiration but not significantly earlier, and, even then, there may be fluid re-accumulation by the time of transfer. Aboulghar et al. reported that aspiration one month prior to retrieval did not improve pregnancy rates [151]. If a hydrosalpinx develops during stimulation, an alternative option is to freeze all embryos and perform a salpingectomy later (Figure 58.16).

Ultrasound-guided IVF procedures: Oocyte retrieval and embryo transfer (ET)

Laparoscopy was the first technique used for oocyte retrieval. The ultrasound guided follicular aspiration was first described in the early 1980s by a Danish group, Lenz and Lauritsen, using abdominal ultrasound [152]. TVUS guided oocyte retrieval is the current standard of care since the late 1980s and is associated with a low complication risk of injury to bladder, bowel, or bleeding from blood vessels. It is usually performed under sedation with a 17-gauge needle. Thinner needles and lower pressure should be used for the *in vitro* maturation technique of aspiration of immature eggs and smaller follicles. The tip of the needle is echogenic and can be visualized at all times and is aligned with the ultrasound beam. The current standard of care for oocyte retrieval is transvaginal aspiration under ultrasound guidance, and there are no randomized controlled trials comparing techniques of transabdominal versus transvaginal approaches. Flushing of follicles has not been shown to make a difference in oocyte yield, takes more time, and should not be routinely performed.

ET is a critical step in ART and can be performed with or without ultrasound guidance. However, Cochrane reviews demonstrated a significant difference between “ultrasound guided” and “clinical touch” methods, so utilizing ultrasound during ET has

become the standard of care [152–154]. The ultrasound guided ET significantly improved clinical pregnancy rate (OR = 1.49; CI 1.29–1.72; $p < 0.00001$) [155–157]. There were no statistical differences for other clinical outcomes such as ectopic pregnancy, miscarriage, or multiple gestation rates. Ultrasound guidance of the transfer catheter resulted in higher pregnancy rates in all but one of the studies identified; this difference was significant in five of the eight studies and the preferred use of the soft catheters was shown. The one study which did not show any difference had a single, very experienced operator. The Cochrane review also compared the incidence of retained embryos for ultrasound versus clinical touch (3.2%–10%) [157]. In one study, it was noted in 35% of the transfers that the catheter touched the top of the uterus and the reduction of retained embryos occurred by US guidance with mid-uterine ET. Retained embryos lead to increased risk of blood on the catheter. Blood on the catheter decreased PR by 3.2%–10%. The majority of programs are using ultrasound guidance and our experience has shown improvement. The ultrasound is especially ideal in training programs with the use of simulation prior to performing real transfers.

The advantages of ultrasound guidance are that the physician can avoid touching the fundus, which has been shown to be deleterious, and can reduce the incidence of difficult transfers by allowing the direction of the catheter along the contour of the cavity and assure embryos are placed properly [158]. Several transfer catheters with echogenic tips have been produced, making it easier to visualize placement, but no significant increase in pregnancy rates has been demonstrated. A few studies comparing 2D versus 3D ultrasound guidance show a possible advantage of 3D in monitoring catheter placement, but this is not commonly used [159]. Gergely et al. [160] first described 1222 women undergoing 3D US-guided ET, reporting encouraging pregnancy rates. In the following years, the authors went on to present an updated cohort ($n = 5073$) where they concluded that since introducing this technique, their centre saw a 10.0% increase in the pregnancy rate and a 1.3% reduction in ectopic pregnancy rate [161].

Despite these preliminary findings from observational studies, a prospective RCT comparing 3D with 2D US-guided ET in 474 women showed no difference in ongoing pregnancy rates between the two groups (35.4% vs 37.1%, $P = 0.70$; rate ratio 0.96, 95% CI 0.75–1.21) [162].

These findings should not however undermine the fact that 3D US-guided ET can be potentially useful in cases with abnormal uterine anatomy, such as bicornuate uteri, and also informative when an apparently uncomplicated ET results in an ectopic pregnancy. Furthermore, although 3D US provides only a static image, further advancement in technology may allow for 3D US to perform live real-time examinations (i.e. 4D US) with the same ease as 2D US real-time examinations, which may prove to be of more value within this context.

Ultrasound for the diagnosis and treatment of ART complications and outcome

Ultrasound for the diagnosis and treatment of ovarian hyperstimulation syndrome (OHSS)

Ovarian hyperstimulation syndrome (OHSS) is a serious iatrogenic condition that arises in women undergoing ovulation induction with fertility medication and occurs during the luteal phase of the ovulatory cycle after hCG trigger, usually peaking three to seven days later or during early pregnancy. The incidence

of severe OHSS ranges from 0.5% to 5%, with increased risks in women with PCOS, thin body habitus, young age, and the use of long GnRH agonist protocols and high oestradiol levels [163]. Preventing OHSS is crucial in ART treatment and strategies to minimize the risk include the use of the GnRH antagonist protocol with the GnRH agonist trigger. It is characterized by VEGF over-expression, ovarian enlargement, and pelvic discomfort. In more severe cases, abdominal distension, nausea, vomiting, and ascites may also occur [164]. In severe cases, the third-spacing of fluid into the peritoneal and pleural cavities leads to respiratory compromise, hypotension, increased intra-abdominal pressure, and renal compromise related to decreased perfusion. The ovaries can enlarge to more than 5–10 cm in diameter, predisposing them to torsion. Sonographic findings in patients with OHSS include markedly enlarged multi-cystic ovaries. Doppler evaluation should always be performed in symptomatic patients to help assess for torsion, although the presence of blood flow does not exclude the diagnosis. Drainage of the ascites for improvement of symptoms is done by abdominal or vaginal approach under ultrasound guidance, and a catheter can be left in the abdominal cavity for drainage at home to avoid hospitalization if there are no electrolyte or renal anomalies [165].

Early pregnancy ultrasound and pregnancy of unknown locations

Ultrasound is essential for the diagnosis of clinical pregnancy, for position of the pregnancy, and number of sacs and fetuses. Recent emphasis has been on reducing the number of embryos transferred to reduce the risk of multiple births. In a normally developing pregnancy, a blastocyst implants by 23 days of menstrual age. The first structure identified by TVUS is the gestational sac (GS), appearing as a spherical, fluid-filled cavity surrounded by an echogenic rim. A double decidual sac sign is a reliable signal of an intrauterine pregnancy. There is a correlation between sac size and hCG level and gestational age, but there is variability, and it is helpful to monitor sequential sonographic milestones. As development progresses, the first structure inside the GS is the yolk sac, followed by the embryo. The yolk sac is a spherical, echogenic ring-like formation with a sonolucent centre, and its presence confirms a true intrauterine pregnancy (IUP) with 100% PPV. The confirmation of yolk sac is necessary by 37–40 menstrual days or six weeks gestation. Fetal heart beat is visible from six weeks and two days gestation based on ET dates. It is seen as a linear echodensity next to the yolk sac. The embryo or fetal pole is measured along its long axis and is called a “crown-rump length” (CRL). Subchorionic haematoma, a fluid collection between the chorionic membrane and decidua, is very common with ART pregnancies and is associated with abnormal placentation and a higher risk of miscarriage. Discriminatory values should be used with caution, as they are a range rather than a specific cut-off value. In a recent study, the discriminatory levels at which structures would be seen 99% of the time were 3510 mIU/mL for a gestational sac, 17,716 mIU/mL for a yolk sac, and 47,685 mIU/mL for a fetal pole [166]. However, threshold values are much lower at 390, 1094, and 1394 mIU/mL for the gestational sac, yolk sac, and fetal pole, respectively. When the ultrasound reveals an IUP, but neither an embryonic pole nor fetal heart activity are identified, the pregnancy is classified as an intrauterine pregnancy of unknown viability. Based on the new criteria, a mean sac diameter (MSD) \geq 16–17 mm with an empty gestational sac is highly suggestive, and MSD $>$ 25 mm is definitive of a failed pregnancy. CRL \geq 5–6 mm with absence of fetal cardiac activity is highly



FIGURE 58.17 Ectopic pregnancy coronal view.

suggestive, and CRL $>$ 7 mm as definitive of a failed pregnancy [167]. In terms of time, there is definitely a failed pregnancy if more than two weeks pass after a gestational sac is seen without a yolk sac and more than 10 days after a gestational sac and yolk sac are identified but an embryo is not. In addition, approximately 90% of incomplete abortions, and 50% of missed abortions, can be expected to spontaneously abort within two weeks of initial presentation and ultrasound [168].

When compared to natural conception, ART increases the chance of multiple gestations. Twinning should be classified as either monozygotic (a single ovum divides into two embryos) or dizygotic (two separate ova) and dichorionic/diamniotic, monochorionic/diamniotic, or monochorionic/monoamniotic. The type of twinning affects the incidence of maternal and fetal morbidity and mortality. A pregnancy of unknown location may require serial ultrasounds. Ultrasonography is the primary diagnostic modality for ectopic pregnancy. The visualization of a fluid-filled sac outside the uterine cavity that contains an embryo or a yolk sac is definitive for ectopic pregnancy. An adnexal mass with the “tubal ring” is also highly predictive. A pregnancy outside the endometrial cavity can be visualized best in the coronal view (Figure 58.17). A series of case reports have highlighted the value of 3D US in the diagnosis of interstitial pregnancies, as the reconstructed coronal view of the uterus allows delineation of the entire uterine cavity in a single plane, and clear identification of the intramural portion of the fallopian tube [169, 170]. Assessment with 3D US may also prove to be of value in cases of cervical/CS ectopic pregnancies, by delineating the uterine cavity and distinguishing it from the cervical canal [171].

The presence of an intrauterine pregnancy in an asymptomatic patient conceived by IVF should not exclude the diagnosis of a concurrent ectopic pregnancy, called a heterotopic pregnancy, so evaluation of the adnexa should be done in all circumstances. In the case of heterotopic pregnancy, methotrexate injection is unacceptable, and laparoscopic surgery or aspiration of the gestational sac and injection with potassium chloride under transvaginal sonography can be done.

Conclusions

Modern ART and infertility treatments cannot be imagined today without ultrasound imaging, and advances in both fields have occurred simultaneously. Ultrasound most certainly

encompasses every aspect in the clinical management of reproductive medicine. The use of 3D visualization of the pelvic structures is the most striking advancement in the use of ultrasound in ART. As costs decrease, accessibility will increase. The future will bring smaller and portable ultrasounds for increased access in underserved communities as well as more standardization and increased automation with savings in time and possible improved outcomes. Attempts have been made to focus on the assessment of female reproductive function by artificial intelligence-aided ultrasound to monitor follicles, assess endometrial receptivity, and predict the pregnancy outcome of IVF-ET [172]. With modern ultrasound usage, we can see better, and do ART better.

References

- Matijevic R, Grgic O. Predictive values of ultrasound monitoring of the menstrual cycle. *Curr Opin Obstet Gynecol.* 2005;17:405–10.
- Jokubkiene L, Sladkevicius P, Rovas L, Valentin L. Assessment of changes in volume and vascularity of the ovaries during the normal menstrual cycle using three-dimensional power Doppler ultrasound. *Hum Reprod.* 2006;21:2661–8.
- Durfee SM, Frates MC. Sonographic spectrum of the corpus luteum in early pregnancy: Gray-scale, color, and pulsed Doppler appearance. *J Clin Ultrasound.* 1999;27:55–9.
- Nelson SM. Biomarkers of ovarian response: Current and future applications. *Fertil Steril.* 2013;99:963–9.
- Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: Implications for forecasting menopause. *Hum Reprod.* 1992;7:1342–6.
- Muasher SJ, Oehninger S, Simonetti S, Matta J, Ellis LM, Liu HC, et al. The value of basal and/or stimulated serum gonadotropin levels in prediction of stimulation response and in vitro fertilization outcome. *Fertil Steril.* 1988;50:298–307.
- Broekmans FJ, Kwee J, Hendriks DJ, Mol BW, Lambalk CB. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update.* 2006;12:685–718.
- Broekmans FJ, Knauff EA, te Velde ER, Macklon NS, Fauser BC. Female reproductive ageing: Current knowledge and future trends. *Trends Endocrinol Metab.* 2007;18:58–65.
- Broekmans FJ, de Ziegler D, Howles CM, Gougeon A, Trew G, Olivennes F. The antral follicle count: Practical recommendations for better standardization. *Fertil Steril.* 2010;94:1044–51.
- Riggs RM, Duran EH, Baker MW, Kimble TD, Hobeika E, Yin L, et al. Assessment of ovarian reserve with anti-mullerian hormone: A comparison of the predictive value of anti-mullerian hormone, follicle-stimulating hormone, inhibin B, and age. *American Journal of Obstetrics and Gynecology.* 2008;199:202 e1–8.
- Hendriks DJ, Mol BW, Bancsi LF, Te Velde ER, Broekmans FJ. Antral follicle count in the prediction of poor ovarian response and pregnancy after in vitro fertilization: A meta-analysis and comparison with basal follicle-stimulating hormone level. *Fertil Steril.* 2005;83:291–301.
- Marca L, Siginolfi A, Radi G, Argento D, Baraldi C, Artenisio E AC, et al. Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). *Hum Reprod Update.* 2010;16.
- Lukaszuk K, Kunicki M, Liss J, Lukaszuk M, Jakiel G. Use of ovarian reserve parameters for predicting live births in women undergoing in vitro fertilization. *Eur J Obstet Gynecol Reprod Biol.* 2013;168:173–7.
- Hansen KR, Hodnett GM, Knowlton N, Craig LB. Correlation of ovarian reserve tests with histologically determined primordial follicle number. *Fertil Steril.* 2011;95:170–5.
- Jayaprakasan K, Al-Hasie H, Jayaprakasan R, Campbell B, Hopkisson J, Johnson I, et al. The three-dimensional ultrasonographic ovarian vascularity of women developing poor ovarian response during assisted reproduction treatment and its predictive value. *Fertil Steril.* 2009;92:1862–9.
- Almog B, Shehata F, Shalom-Paz E, Tan SL, Tulandi T. Age-related nomogram for antral follicle count: McGill reference guide. *Fertil Steril.* 2011;95:663–6.
- Iliodromiti S, Anderson RA, Nelson SM. Technical and performance characteristics of anti-mullerian hormone and antral follicle count as biomarkers of ovarian response. *Hum Reprod Update.* 2015;21:698–710.
- Scheffer GJ, Broekmans FJ, Loosman CW, Blankenstein M, Fauser BC, te Jong FH, et al. The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. *Hum Reprod.* 2003;18:700–6.
- Raine-Fenning N, Jayaprakasan K, Chamberlain S, Devlin L, Priddle H, Johnson I. Automated measurements of follicle diameter: A chance to standardize? *Fertil Steril.* 2009;91:1469–72.
- Stadtmauer LA, Tur-Kaspa I. Ovarian reserve and ovarian cysts. In: Stadtmauer LA, Tur-Kaspa I, eds. *Ultrasound Imaging in Reproductive Medicine: Advances in Infertility Work-up, Treatment, and ART.* New York: Springer, 2014:xvii, 360 pages.
- Firouzabadi RD, Sekhavat L, Javedani M. The effect of ovarian cyst aspiration on IVF treatment with GnRH. *Arch Gynecol Obstet.* 2010;281:545–9.
- Raine-Fenning N, Jayaprakasan K, Deb S. Three-dimensional ultrasonographic characteristics of endometrioma. *Ultrasound Obstet Gynecol.* 2008;31:718.
- González-Foruria I, Soldevila PB, Rodríguez I, Rodríguez-Purata J, Pardos C, García S, Pascual M, Barri PN, Polyzos NP. Do ovarian endometriomas affect ovarian response to ovarian stimulation for IVF/ICSI? *Reprod Biomed Online.* 2020;41(1):37–43.
- Ferrero S, Scala C, Tafi E, Racca A, Venturini PL, Leone Roberti Maggiore U. Impact of large ovarian endometriomas on the response to superovulation for in vitro fertilization: A retrospective study. *Eur J Obstet Gynecol Reprod Biol.* 2017;213:17–21.
- Senapati S, Sammel MD, Morse C, Barnhart KT. Impact of endometriosis on in vitro fertilization outcomes: An evaluation of the society for assisted reproductive technologies database. *Fertil Steril.* 2016;106(1):164–171.e1. doi: [10.1016/j.fertnstert.2016.03.037](https://doi.org/10.1016/j.fertnstert.2016.03.037).
- Tao X, Chen L, Ge S, Cai L. Weigh the pros and cons to ovarian reserve before stripping ovarian endometriomas prior to IVF/ICSI: A meta-analysis. *PLoS One.* 2017;12(6):e0177426. doi: [10.1371/journal.pone.0177426](https://doi.org/10.1371/journal.pone.0177426).
- Wong BC, Gillman NC, Oehninger S, Gibbons WE, Stadtmauer LA. Results of in vitro fertilization in patients with endometriomas: Is surgical removal beneficial? *Am J Obstet Gynecol.* 2004;191:597–606; discussion -7.
- Caspi B, Weissman A, Zalel Y, Barash A, Tulandi T, Shoham Z. Ovarian stimulation and in vitro fertilization in women with mature cystic teratomas. *Obstet Gynecol.* 1998;92:979–81.
- Alborzi S, Khodaei R, Parsanejad ME. Ovarian size and response to laparoscopic ovarian electro-cauterization in polycystic ovarian disease. *Int J Gynaecol Obstet.* 2001;74:269–74.
- Amer SA, Li TC, Bygrave C, Sprigg A, Saravelos H, Cooke ID. An evaluation of the inter-observer and intra-observer variability of the ultrasound diagnosis of polycystic ovaries. *Hum Reprod.* 2002;17:1616–22.
- Ardaens Y, Robert Y, Lemaitre L, Fossati P, Dewailly D. Polycystic ovarian disease: Contribution of vaginal endosonography and reassessment of ultrasonic diagnosis. *Fertil Steril.* 1991;55:1062–8.
- Jonard S, Robert Y, Cortet-Rudelli C, Pigny P, Decanter C, Dewailly D. Ultrasound examination of polycystic ovaries: Is it worth counting the follicles? *Hum Reprod.* 2003;18:598–603.
- Rotterdam EA-SPCWG. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril.* 2004;81:19–25.

34. Rotterdam EA-SPcwg. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod.* 2004;19:41–7.
35. Teede H, Misso M, Costello M, et al. ASRM recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome on behalf of the International PCOS Network. *Fertil Steril.* 2018;110:364–79.
36. Kyei-Mensah A, Maconochie N, Zaidi J, Pittrof R, Campbell S, Tan SL. Transvaginal three-dimensional ultrasound: Reproducibility of ovarian and endometrial volume measurements. *Fertil Steril.* 1996;66:718–22.
37. Onofriescu A, Bors A, Luca A, Holcov M, Onofriescu M, Vulpoi C. GnRH antagonist IVF protocol in PCOS. *Curr Health Sci J.* 2013;39(1):20–5.
38. Baerwald A, Pierson R. Ovarian follicular waves during the menstrual cycle: Physiologic insights into novel approaches for ovarian stimulation. *Fertil Steril.* 2020;114(3):443–57.
39. Hackeloer BJ, Robinson HP. Ultrasound examination of the growing ovarian follicle and of the corpus luteum during the normal physiologic menstrual cycle (author's transl). *Geburtshilfe Frauenheilkd.* 1978;38:163–8.
40. Kwan I, Bhattacharya S, McNeil A, van Rumste MM. Monitoring of stimulated cycles in assisted reproduction (IVF and ICSI). The Cochrane Database of Systematic Reviews 2008;CD005289.
41. Revelli A, Martiny G, Delle Piane L, Benedetto C, Rinaudo P, Tur-Kaspa I. A critical review of bi-dimensional and three-dimensional ultrasound techniques to monitor follicle growth: Do they help improving IVF outcome? *Reprod Biol Endocrinol.* 2014;12:107.
42. Deb S, Jayaprakasan K, Campbell BK, Clewes JS, Johnson IR, Raine-Fenning NJ. Intraobserver and interobserver reliability of automated antral follicle counts made using three-dimensional ultrasound and SonoAVC. *Ultrasound Obstet Gynecol.* 2009;33:477–83.
43. Raine-Fenning N, Jayaprakasan K, Clewes J, Joergner I, Bonaki SD, Chamberlain S, et al. SonoAVC: A novel method of automatic volume calculation. *Ultrasound Obstet Gynecol.* 2008;31:691–6.
44. Ata B, Tulandi T. Ultrasound automated volume calculation in reproduction and in pregnancy. *Fertil Steril.* 2011;95:2163–70.
45. Deutch TD, Joergner I, Matson DO, Oehninger S, Bocca S, Hoenigmann D, et al. Automated assessment of ovarian follicles using a novel three-dimensional ultrasound software. *Fertil Steril.* 2009;92:1562–8.
46. Raine-Fenning N, Deb S, Jayaprakasan K, Clewes J, Hopkisson J, Campbell B. Timing of oocyte maturation and egg collection during controlled ovarian stimulation: A randomized controlled trial evaluating manual and automated measurements of follicle diameter. *Fertil Steril.* 2010;94:184–8.
47. Noor N, Vignarajan P, Malhotra N, Vanamail P. Three-dimensional automated volume calculation (Sonography-based automated volume count) versus two-dimensional manual ultrasonography for follicular tracking and oocyte retrieval in women undergoing in vitro fertilization–embryo transfer: A randomized controlled trial. *J Hum Reprod Sci.* 2020;13(4):296–302.
48. Hernández J, Rodríguez-Fuentes A, Puopolo M, Palumbo A. Follicular volume predicts 315 oocyte maturity: A prospective cohort study using three-dimensional ultrasound and 316 SonoAVC. *Reprod Sci.* 2016;23(12):1639–43.48.
49. Vandekerckhove F, Bracke V, De Sutter P. The value of automated follicle volume measurements in IVF/ICSI. *Front Surg.* 2014;1:18.
50. Casper RF. It's time to pay attention to the endometrium. *Fertil Steril.* 2011;96:519–21.
51. Raine-Fenning NJ, Campbell BK, Clewes JS, Kendall NR, Johnson IR. Defining endometrial growth during the menstrual cycle with three-dimensional ultrasound. *BJOG.* 2004a;111:944–9.
52. Raga F, Bonilla-Musoles F, Casan EM, Klein O, Bonilla F. Assessment of endometrial volume by three-dimensional ultrasound prior to embryo transfer: Clues to endometrial receptivity. *Hum Reprod.* 1999;14:2851–4.
53. Kovachev E, Ganchev Z, Cherneva S, Zokhav E, Shperberg A. Measurement of endometrial volume and endometrial thickness for assessment of endometrial receptivity in assisted reproductive techniques. *Akush Ginekol (Mosk).* 2005;44:27–33.
54. Kupesic S, Bekavac I, Bjelos D, Kurjak A. Assessment of endometrial receptivity by transvaginal color Doppler and three-dimensional power Doppler ultrasonography in patients undergoing in vitro fertilization procedures. *J Ultrasound Med.* 2001;20:125–34.
55. Friedler S, Schenker JG, Herman A, Lewin A. The role of ultrasonography in the evaluation of endometrial receptivity following assisted reproductive treatments: A critical review. *Hum Reprod Update.* 1996;2:323–35.
56. Merce LT, Barco MJ, Bau S, Troyano J. Are endometrial parameters by three-dimensional ultrasound and power Doppler angiography related to in vitro fertilization/embryo transfer outcome? *Fertil Steril.* 2008;89:111–7.
57. Killick SR. Ultrasound and the receptivity of the endometrium. *Reprod Biomed Online.* 2007;15:63–7.
58. Randolph JF Jr., Ying YK, Maier DB, Schmidt CL, Riddick DH. Comparison of real-time ultrasonography, hysterosalpingography, and laparoscopy/hysteroscopy in the evaluation of uterine abnormalities and tubal patency. *Fertil Steril.* 1986;46:828–32.
59. de Kroon CD, de Bock GH, Dieben SW, Jansen FW. Saline contrast hysterosonography in abnormal uterine bleeding: A systematic review and meta-analysis. *BJOG.* 2003;110:938–47.
60. Brown SE, Coddington CC, Schnorr J, Toner JP, Gibbons W, Oehninger S. Evaluation of outpatient hysteroscopy, saline infusion hysterosonography, and hysterosalpingography in infertile women: A prospective, randomized study. *Fertil Steril.* 2000;74:1029–34.
61. American College of O, Gynecologists. ACOG technology assessment in obstetrics and gynecology no. 5: Sonohysterography. *Obstet Gynecol.* 2008;112 (6):1467–9.
62. Spieldoch RL, Winter TC, Schouweiler C, Ansay S, Evans MD, Lindheim SR. Optimal catheter placement during sonohysterography: A randomized controlled trial comparing cervical to uterine placement. *Obstet Gynecol.* 2008;111:15–21.
63. Tur-Kaspa I, SL. Sonohysterography (SHG) in reproductive medicine. In: Stadtmauer LA, Tur-Kaspa I, eds. *Ultrasound Imaging in Reproductive Medicine: Advances in Infertility Work-up, Treatment, and ART.* New York: Springer, 2014:xvii, 360 pages.
64. Werbrouck E, Veldman J, Luts J, Van Huffel S, Van Schoubroeck D, Timmerman D, et al. Detection of endometrial pathology using saline infusion sonography versus gel instillation sonography: A prospective cohort study. *Fertil Steril.* 2011;95:285–8.
65. de Ziegler D. Contrast ultrasound: A simple-to-use phase-shifting medium offers saline infusion sonography-like images. *Fertil Steril.* 2009;92:369–73.
66. Soares SR, Barbosa dos Reis MM, Camargos AF. Diagnostic accuracy of sonohysterography, transvaginal sonography, and hysterosalpingography in patients with uterine cavity diseases. *Fertil Steril.* 2000;73:406–11.
67. Makris N, Skartados N, Kalmantis K, Mantzaris G, Papadimitriou A, Antsaklis A. Evaluation of abnormal uterine bleeding by transvaginal 3-d hysterosonography and diagnostic hysteroscopy. *Eur J Gynaecol Oncol.* 2007;28:39–42.
68. Tur-Kaspa I, Gal M, Hartman M, Hartman J, Hartman A. A prospective evaluation of uterine abnormalities by saline infusion sonohysterography in 1,009 women with infertility or abnormal uterine bleeding. *Fertil Steril.* 2006;86:1731–5.
69. Alborzi S, Parsanezhad ME, Mahmoodian N, Alborzi S, Alborzi M. Sonohysterography versus transvaginal sonography for screening of patients with abnormal uterine bleeding. *Int J Gynaecol Obstet.* 2007;96:20–3.
70. Sylvestre C, Child TJ, Tulandi T, Tan SL. A prospective study to evaluate the efficacy of two- and three-dimensional sonohysterography in women with intrauterine lesions. *Fertil Steril.* 2003;79:1222–5.

71. Acholou UC, Silberzweig J, Stein DE, Keltz M. Hysterosalpingography versus sono- hysterosalpingography for intrauterine abnormalities. *J SLS.* 2011;15:471–4.
72. Seshadri S, El-Toukhy T, Douiri A, Jayaprakasan K, Khalaf Y. Diagnostic accuracy of saline infusion sonography in the evaluation of uterine cavity abnormalities prior to assisted reproductive techniques: A systematic review and meta-analyses. *Hum Reprod Update* 2015;21:262–274. doi: [10.1093/humupd/dmu057](https://doi.org/10.1093/humupd/dmu057).
73. Nieuwenhuis LL, Hermans FJ, Bij de Vaate AJM, Leeflang MM, Brölmann HA, Hennenkamp WJ, Mol BWJ, Clark TJ, Huirne JA. Three-dimensional saline infusion sonography compared to two-dimensional saline infusion sonography for the diagnosis of focal intracavitary lesions. *Cochrane Database Syst Rev.* 2017;5(5): CD011126. doi: [10.1002/14651858.CD011126.pub2](https://doi.org/10.1002/14651858.CD011126.pub2).
74. Shavell VI, Thakur M, Sawant A, Kruger ML, Jones TB, Singh M, et al. Adverse obstetric outcomes associated with sonographically identified large uterine fibroids. *Fertil Steril.* 2012;97: 107–10.
75. Klatsky PC, Tran ND, Caughey AB, Fujimoto VY. Fibroids and reproductive outcomes: A systematic literature review from conception to delivery. *American Journal of Obstetrics and Gynecology.* 2008;198:357–66.
76. Salim R, Lee C, Davies A, Jolaoso B, Ofuasia E, Jurkovic D. A comparative study of three-dimensional saline infusion sonohysterography and diagnostic hysteroscopy for the classification of submucous fibroids. *Hum Reprod.* 2005;20:253–7.
77. Mayer DP, Shipilov V. Ultrasonography and magnetic resonance imaging of uterine fibroids. *Obstet Gynecol Clin North Am.* 1995;22:667–725.
78. Sheth S, Macura K. Sonography of the uterine myometrium: Myomas and beyond. *Ultrasound Clinics.* 2007;2:267–95.
79. Stadtmauer L, AM, Oehninger S. Efficacy of 40 cases of robot-assisted laparoscopic myomectomy in women with symptomatic fibroids and infertility. *J Min Invasive Surg.* 2010.
80. Pritts EA, Parker WH, Olive DL. Fibroids And infertility: An updated systematic review of the evidence. *Fertil Steril.* 2009;91:1215–23.
81. Pritts EA. Fibroids and infertility: A systematic review of the evidence. *Obstet Gynecol Surv.* 2001;56:483–91.
82. Metwally M, Raybould G, Cheong YC, Horne AW. Surgical treatment of fibroids for subfertility. *Cochrane Database Syst Rev.* 2020 Jan 29;1(1):CD003857. doi: [10.1002/14651858.CD003857.pub4](https://doi.org/10.1002/14651858.CD003857.pub4).
83. Sunkara SK, Khairy M, El-Toukhy T, Khalaf Y, Coomarasamy A. The effect of intramural fibroids without uterine cavity involvement on the outcome of IVF treatment: A systematic review and meta-analysis. *Hum Reprod.* 2010;25:418–29.
84. Yan L, Ding L, Li C, Wang Y, Tang R, Chen ZJ. Effect of fibroids not distorting the endometrial cavity on the outcome of in vitro fertilization treatment: A retrospective cohort study. *Fertil Steril.* 2014;101:716–21.
85. Somigliana E, De Benedictis S, Vercellini P, Nicolosi AE, Benaglia L, Scarduelli C, Ragni G, Fedele L. Fibroids not encroaching the endometrial cavity and IVF success rate: A prospective study. *Hum Reprod.* 2011;26:834–9.
86. Rajan DK, Margau R, Kroll RR, Simons ME, Tan KT, Jaskolka JD, et al. Clinical utility of ultrasound versus magnetic resonance imaging for deciding to proceed with uterine artery embolization for presumed symptomatic fibroids. *Clin Radiol.* 2011;66:57–62.
87. Rabinovici J, David M, Fukunishi H, Morita Y, Gostout BS, Stewart EA, et al. Pregnancy outcome after magnetic resonance-guided focused ultrasound surgery (MRgFUS) for conservative treatment of uterine fibroids. *Fertil Steril.* 2010;93:199–209.
88. Meredith SM, Sanchez-Ramos L, Kaunitz AM. Diagnostic accuracy of transvaginal sonography for the diagnosis of adenomyosis: Systematic review and metaanalysis. *Am J Obstet Gynecol.* 2009;201:107.e1–6.
89. Marshburn PB, Hurst BS. Disorders of Menstruation. Chichester, West Sussex; Hoboken, NJ: Wiley-Blackwell, 2011.
90. Dueholm M. Transvaginal ultrasound for diagnosis of adenomyosis: A review. *Best Pract Res Clin Obstet Gynaecol.* 2006;20:569–82.
91. Champaneria R, Abedin P, Daniels J, Balogun M, Khan KS. Ultrasound scan and magnetic resonance imaging for the diagnosis of adenomyosis: Systematic review comparing test accuracy. *Acta Obstet Gynecol Scand.* 2010;89:1374–84.
92. Sharma K, Bora MK, Venkatesh BP, Barman P, Roy SK, Jayagurunathan U, Sellamuthu E, Moidu F. Role of 3D ultrasound and Doppler in differentiating clinically suspected cases of leiomyoma and adenomyosis of uterus. *J Clin Diagn Res.* 2015;9: QC08–12.
93. Maheshwari A, Gurunath S, Fatima F, Bhattacharya S. Adenomyosis and subfertility: A systematic review of prevalence, diagnosis, treatment and fertility outcomes. *Hum Reprod Update.* 2012;18:374–92.
94. Galliano D, Bellver J, Diaz-Garcia C, Simon C, Pellicer A. ART and uterine pathology: How relevant is the maternal side for implantation? *Hum Reprod Update.* 2015;21:13–38.
95. American Association of Gynecologic Laparoscopists. AAGL practice report: practice guidelines for the diagnosis and management of endometrial polyps. *J Min Invasive Gynecol.* 2012;19:3–10.
96. Inoue T, Kitajima M, Taniguchi K, Masuzaki H. Three-dimensional saline-infusion sonohysterography is useful for the identification of endometrial polyp. *J Obstet Gynaecol Res.* 2016;42(7):855–9. doi: [10.1111/jog.12994](https://doi.org/10.1111/jog.12994).
97. Kupesic S, Kurjak A, Skenderovic S, Bjelos D. Screening for uterine abnormalities by three-dimensional ultrasound improves perinatal outcome. *J Perinat Med.* 2002;30:9–17.
98. Tiras B, Korucuoglu U, Polat M, Zeyneloglu HB, Saltik A, Yarali H. Management of endometrial polyps diagnosed before or during ICSI cycles. *Reprod Biomed Online.* 2012;24:123–8.
99. Lass A, Williams G, Abusheikha N, Brinsden P. The effect of endometrial polyps on outcomes of in vitro fertilization (IVF) cycles. *J Assist Reprod Genet.* 1999;16:410–5.
100. Bosteels J, van Wessel S, Weyers S, Broekmans FJ, D'Hooghe TM, Bongers MY, Mol BWJ. Hysteroscopy for treating subfertility associated with suspected major uterine cavity abnormalities. *Cochrane Database Syst Rev.* 2018;12(12):CD009461. doi: [10.1002/14651858.CD009461.pub4](https://doi.org/10.1002/14651858.CD009461.pub4).
101. Vitale SG, Haimovich S, Laganà AS, Alonso L, Di Spiezo Sardo A, Carugno J; From the Global Community of Hysteroscopy Guidelines Committee. Endometrial polyps. An evidence-based diagnosis and management guide. *Eur J Obstet Gynecol Reprod Biol.* 2021 May;260:70–77. doi: [10.1016/j.ejogrb.2021.03.017](https://doi.org/10.1016/j.ejogrb.2021.03.017).
102. Bacelar AC, Wilcock D, Powell M, Worthington BS. The value of MRI in the assessment of traumatic intra-uterine adhesions (Asherman's syndrome). *Clin Radiol.* 1995;50:80–3.
103. Knopman J, Copperman AB. Value of 3D ultrasound in the management of suspected Asherman's syndrome. *J Reprod Med.* 2007;52:1016–22.
104. Kodaman PH, Arici A. Intra-uterine adhesions and fertility outcome: How to optimize success? *Curr Opin Obstet Gynecol.* 2007;19:207–14.
105. Diamond MP, Freeman ML. Clinical implications of postsurgical adhesions. *Hum Reprod Update.* 2001;7:567–76.
106. Pace S, Stentella P, Catania R, Palazzetti PL, Frega A. Endoscopic treatment of intrauterine adhesions. *Clin Exp Obstet Gynecol.* 2003;30:26–8.
107. Sakhel K, Benson CB, Platt LD, Goldstein SR, Benacerraf BR. Begin with the basics: Role of 3-dimensional sonography as a first-line imaging technique in the cost-effective evaluation of gynecologic pelvic disease. *J Ultrasound Med.* 2013;32:381–8.
108. Balen FG, Allen CM, Gardener JE, Siddall NC, Lees WR. 3-dimensional reconstruction of ultrasound images of the uterine cavity. *Br J Radiol.* 1993;66:588–91.
109. Jurkovic D, Gruboeck K, Tailor A, Nicolaides KH. Ultrasound screening for congenital uterine anomalies. *Br J Obstet Gynaecol.* 1997;104:1320–1.

110. Trolice M. The septate uterus and metroplasty-another dogma under siege. *Fertil Steril.* 2021;116:693–4.
111. Bhagavath B, Lindheim SR. Optimal management of symptomatic cesarean scar defects. *Fertil Steril.* 2018;110(3):417–8. doi: [10.1016/j.fertnstert.2018.06.035](https://doi.org/10.1016/j.fertnstert.2018.06.035).
112. Gurol-Urganci I, Bou-Antoun S, Lim CP, Cromwell DA, Mahmood TA, Templeton A, van der Meulen JH. Impact of caesarean section on subsequent fertility: A systematic review and meta-analysis. *Hum Reprod.* 2013;28:1943–52.
113. Baranov A, Gunnarsson G, Salvesen KA, Isberg PE, Vikhareva O. Assessment of cesarean hysterotomy scar in non-pregnant women: Reliability of transvaginal sonography with and without contrast enhancement. *Ultrasound Obstet Gynecol.* 2016;47:499–505.
114. Marjolein Bij de Vaate AJ, Linskens IH, van der Voet LF, Twisk JW, Brolmann HA, Huirne JA. Reproducibility of three-dimensional ultrasound for the measurement of a niche in a caesarean scar and assessment of its shape. *Eur J Obstet Gynecol Reprod Biol.* 2015;188:39–44.
115. Glavind J, Madsen LD, Uldbjerg N, Dueholm M. Cesarean section scar measurements in non-pregnant women using three-dimensional ultrasound: A repeatability study. *Eur J Obstet Gynecol Reprod Biol.* 2016;201:65–9.
116. Vervoort A, van der Voet LF, Hennenkamp W, Therkow AL, van Kesteren P, Quartero H, et al. Hysteroscopic resection of a uterine caesarean scar defect (niche) in women with postmenstrual spotting: A randomised controlled trial. *BJOG.* 2018;125: 326–34.
117. Vervoort A, Vissers J, Hennenkamp W, Broelmann H, Huirne J. The effect of laparoscopic resection of large niches in the uterine caesarean scar on symptoms, ultrasound findings and quality of life: A prospective cohort study. *BJOG.* 2018;125:317–25.
118. Smith B, Porter R, Ahuja K, Craft I. Ultrasonic assessment of endometrial changes in stimulated cycles in an in vitro fertilization and embryo transfer program. *Journal of In Vitro Fertilization and Embryo Transfer: IVF.* 1984;1:233–8.
119. Weckstein LN, Jacobson A, Galen D, Hampton K, Hammel J. Low-dose aspirin for oocyte donation recipients with a thin endometrium: Prospective, randomized study. *Fertil Steril.* 1997;68:927–30.
120. Sher G, Fisch JD. Effect of vaginal sildenafil on the outcome of in vitro fertilization (IVF) after multiple IVF failures attributed to poor endometrial development. *Fertil Steril.* 2002;78:1073–6.
121. Liu KE, Hartman M, Hartman A, Luo ZC, Mahutte N. The impact of a thin endometrial lining on fresh and frozen-thaw IVF outcomes: An Analysis of over 40 000 embryo transfers. *Hum Reprod.* 2018;33:1883–8.
122. Shakerian B, Turkogeldi E, Yildiz S, Keles I, Ata B. Endometrial thickness is not predictive for live birth after embryo transfer, even without a cutoff. *Fertil Steril.* 2021;116(1):130–137. doi: [10.1016/j.fertnstert.2021.02.041](https://doi.org/10.1016/j.fertnstert.2021.02.041).
123. Groenewoud ER, Cohlen BJ, Al-Oraiby A, Brinkhuis EA, Broekmans FJM, de Bruin JP, et al. Influence of endometrial thickness on pregnancy rates in modified natural cycle frozen-thawed embryo transfer. *Acta Obstet Gynecol Scand.* 2018;97: 808–15.
124. Al-Ghamdi A, Coskun S, Al-Hassan S, Al-Rejjal R, Awartani K. The correlation between endometrial thickness and outcome of in vitro fertilization and embryo transfer (IVF-ET) outcome. *Reprod Biol Endocrinol.* 2008;6:37.
125. Weissman A, Gotlieb L, Casper RF. The detrimental effect of increased endometrial thickness on implantation and pregnancy rates and outcome in an in vitro fertilization program. *Fertil Steril.* 1999;71:147–9.
126. Dickey RP, Olar TT, Taylor SN, Curole DN, Matulich EM. Relationship of endometrial thickness and pattern to fecundity in ovulation induction cycles: Effect of clomiphene citrate alone and with human menopausal gonadotropin. *Fertil Steril.* 1993;59:756–60.
127. Zohav E, Orvieto R, Anteby EY, Segal O, Melter S, Tur-Kaspa I. Low endometrial volume may predict early pregnancy loss in women undergoing in vitro fertilization. *J Assist Reprod Genet.* 2007;24:259–61.
128. Sundstrom P. Establishment of a successful pregnancy following in-vitro fertilization with an endometrial thickness of no more than 4 mm. *Hum Reprod.* 1998;13:1550–2.
129. Silva Martins R, Helio Oliani A, Vaz Oliani D, Martinez de Oliveira J. Subendometrial resistance and pulsatility index assessment of endometrial receptivity in assisted reproductive technology cycles. *Reprod Biol Endocrinol.* 2019;17(1):62.
130. Kupesic S, Bekavac I, Bjelos D, Kurjak A. Assessment of endometrial receptivity by transvaginal color Doppler and three-dimensional power Doppler ultrasonography in patients undergoing in vitro fertilization procedures. *J Ultrasound Med.* 2001;20:125–34.
131. Chien L-W, Au H-K, Chen P-L, Xiao J, Tzeng C-R. Assessment of uterine receptivity by the endometrial-subendometrial blood flow distribution pattern in women undergoing in vitro fertilization-embryo transfer. *Fertil Steril.* 2002;78:245–51.
132. Maugey-Laulom B, Commenges-Ducos M, Jullien V, Papaxanthos-Roche A, Scotet V, Commenges D. Endometrial vascularity and ongoing pregnancy after IVF. *Eur J Obstet Gynecol Reprod Biol.* 2002;104:137–43.
133. Ng EHY, Chan CCW, Tang OS, Yeung WSB, Ho PC. The role of endometrial and subendometrial blood flows measured by three-dimensional power Doppler ultrasound in the prediction of pregnancy during IVF treatment. *Hum Reprod.* 2006;21:164–70.
134. Sadek S, Matitashvili T, Kovac A, Ramadan H, Stadtmauer L. Assessment of uterine receptivity by endometrial and sub-endometrial blood flow using SlowflowHD in hormone prepared frozen embryo transfer cycles: A pilot study. *J Assist Reprod Genet.* 2022;39(5):1069–79. doi: [10.1007/s10815-022-02454-8](https://doi.org/10.1007/s10815-022-02454-8).
135. Ng EH, Chan CC, Tang OS, Yeung WS, Ho PC. The role of endometrial and subendometrial vascularity measured by three-dimensional power Doppler ultrasound in the prediction of pregnancy during frozen-thawed embryo transfer cycles. *Hum Reprod.* 2006;21:1612–7.
136. Tur-Kaspa I. Fear no pain: Uterine cavity and tubal patency assessment tests should be pain free. *Ultrasound Obstet Gynecol.* 2012;39:247–51.
137. Radic V, Canic T, Valetic J, Duic Z. Advantages and disadvantages of hysterosalpingography in the assessment of the reproductive status of uterine cavity and fallopian tubes. *Eur J Radiol.* 2005;53:268–73.
138. Richman TS, Visconti GN, deCherney A, Polan ML, Alcebo LO. Fallopian tubal patency assessed by ultrasound following fluid injection. Work in progress. *Radiology.* 1984;152:507–10.
139. Balen FG, Allen CM, Siddle NC, Lees WR. Ultrasound contrast hysterosalpingography—evaluation as an outpatient procedure. *Br J Radiol.* 1993;66:592–9.
140. Dietrich M, Suren A, Hinney B, Osmers R, Kuhn W. Evaluation of tubal patency by hysterocontrast sonography (HyCoSy, echovist) and its correlation with laparoscopic findings. *J Clin Ultrasound.* 1996;24:523–7.
141. Deichert U, Schleif R, van de Sandt M, Juhnke I. Transvaginal hysterosalpingo-contrast-sonography (hy-co-sy) compared with conventional tubal diagnostics. *Hum Reprod.* 1989;4:418–24.
142. Chenia F, Hofmeyr GJ, Moolla S, Oratis P. Sonographic hydrotubation using agitated saline: A new technique for improving fallopian tube visualization. *Br J Radiol.* 1997;70:833–6.
143. Saunders RD, Shwayder JM, Nakajima ST. Current methods of tubal patency assessment. *Fertil Steril.* 2011;95:2171–9.
144. van Welie N, van Rijswijk J, Dreyer K, van Hooff MHA, Bruin JP, Verhoeve HR, et al. Can hysterosalpingo-foam sonography replace hysterosalpingography as first-choice tubal patency test? A randomized non-inferiority trial. *Hum Reprod.* 2022 Feb 27:deac034. doi: [10.1093/humrep/deac034](https://doi.org/10.1093/humrep/deac034).

145. Sladkevicius P, Ojha K, Campbell S, Nargund G. Three-dimensional power Doppler imaging in the assessment of fallopian tube patency. *Ultrasound Obstet Gynecol.* 2000;16:644–7.
146. Chan CC, Ng EH, Tang OS, Chan KK, Ho PC. Comparison of three-dimensional hysterosalpingo-contrast-sonography and diagnostic laparoscopy with chromoperturbation in the assessment of tubal patency for the investigation of subfertility. *Acta Obstet Gynecol Scand.* 2005;84:909–13.
147. Kupesic S, Plavsic BM. 2D and 3D hysterosalpingo-contrast-sonography in the assessment of uterine cavity and tubal patency. *Eur J Obstet Gynecol Reprod Biol.* 2007;133:64–9.
148. Johnson NP, Mak W, Sowter MC. Surgical treatment for tubal disease in women due to undergo in vitro fertilisation. *Cochrane Data Syst Rev.* 2004;CD002125.
149. Sills ES, Walsh DJ, Jones CA, Wood SH. Endometrial fluid associated with Essure implants placed before in vitro fertilization: Considerations for patient counseling and surgical management. *Clinical and Experimental Reproductive Medicine.* 2015;42:126–9.
150. Hammadeh N, Coomarasamy A, Ola B, Papaioannou S, Afnan M, Sharif K. Ultrasound-guided hydrosalpinx aspiration during oocyte collection improves pregnancy outcome in IVF: A randomized controlled trial. *Hum Reprod.* 2008;23:1113–7.
151. Aboulghar MA, Mansour RT, Serour GI. Spontaneous intrauterine pregnancy following salpingectomy for a unilateral hydrosalpinx. *Hum Reprod.* 2002;17:1099–100.
152. Lenz S, Lauritsen JG. Ultrasonically guided percutaneous aspiration of human follicles under local anesthesia: A new method of collecting oocytes for in vitro fertilization. *Fertil Steril.* 1982;38:673–7.
153. Mirkin S, Jones EL, Mayer JF, Stadtmauer L, Gibbons WE, Oehninger S. Impact of transabdominal ultrasound guidance on performance and outcome of transcervical uterine embryo transfer. *J Assist Reprod Genet.* 2003;20:318–22.
154. Anderson RE, Nugent NL, Gregg AT, Nunn SL, Behr BR. Transvaginal ultrasound-guided embryo transfer improves outcome in patients with previous failed in vitro fertilization cycles. *Fertil Steril.* 2002;77:769–75.
155. Buckett WM. A meta-analysis of ultrasound-guided versus clinical touch embryo transfer. *Fertil Steril.* 2003;80:1037–41.
156. Brown JA, Buckingham K, Abou-Setta A, Buckett W. Ultrasound versus ‘clinical touch’ for catheter guidance during embryo transfer in women. *Cochrane Data Syst Rev.* 2007;CD006107.
157. Derk RS, Farquhar C, Mol BW, Buckingham K, Heineman MJ. Techniques for preparation prior to embryo transfer. *Cochrane Data Syst Rev.* 2009;CD007682.1
158. Confino E Jr., Tur-Kaspa I. Ultrasound role in embryo transfers. In: Stadtmauer LA, Tur-Kaspa I, eds. *Ultrasound Imaging in Reproductive Medicine: Advances in Infertility Work-up, Treatment, and ART.* New York: Springer, 2014:xvii, 360 pages.
159. Letterie GS. Three-dimensional ultrasound-guided embryo transfer: A preliminary study. *Am J Obstet Gynecol.* 2005;192:1983–7; discussion 7–8.
160. Gergely RZ, DeUgarte CM, Danzer H, Surrey M, Hill D, DeCherney AH. Three dimensional/four dimensional ultrasound-guided embryo transfer using the maximal implantation potential point. *Fertil Steril.* 2005;84:500–3.
161. Gergely RZ. 3D/4D ultrasound-guided embryo transfer targeting maximum implantation potential point increases pregnancy rate, reduces complications. *Reprod Biomed Online.* 2010;20:7.
162. Saravelos SH, Li TC. Ultrasound-guided treatment of intrauterine adhesions in the outpatient setting. *Ultrasound Obstet Gynecol.* 2017;50(2):278–280. doi: [10.1002/uog.16218](https://doi.org/10.1002/uog.16218).
163. Delvigne A, Dubois M, Battheu B, Bassil S, Meuleman C, De Sutter P, et al. The ovarian hyperstimulation syndrome in in-vitro fertilization: A Belgian multicentric study. II. Multiple discriminant analysis for risk prediction. *Hum Reprod.* 1993;8:1361–6.
164. Aboulghar MA, Mansour RT. Ovarian hyperstimulation syndrome: Classifications and critical analysis of preventive measures. *Hum Reprod Update.* 2003;9:275–89.
165. Gebril A, Hamoda H, Mathur R. Outpatient management of severe ovarian hyperstimulation syndrome: A systematic review and A review of existing guidelines. *Hum Fertil (Camb).* 2018;21(2):98–105. doi: [10.1080/14647273.2017.1331048](https://doi.org/10.1080/14647273.2017.1331048).
166. Connolly A, Ryan DH, Stuebe AM, Wolfe HM. Reevaluation of discriminatory and threshold levels for serum beta-hCG in early pregnancy. *Obstet Gynecol.* 2013;121:65–70.
167. Doubilet PM, Benson CB, Bourne T, Blaivas M, Society of Radiologists in Ultrasound Multispecialty Panel on Early First Trimester Diagnosis of Miscarriage and Exclusion of a Viable Intrauterine Pregnancy, et al. Diagnostic criteria for nonviable pregnancy early in the first trimester. *N Engl J Med.* 2013;369:1443–51.
168. Whitworth M, Bricker L, Neilson JP, Dowswell T. Ultrasound for fetal assessment in early pregnancy. *Cochrane Data Syst Rev.* 2010;CD007058.
169. Tanaka Y, Mimura K, Kanagawa T, Kajimoto E, Takahashi K, Kakigano A, Fujita S, Kinugasa-Taniguchi Y, Endo M, Kimura T. Three-dimensional sonography in the differential diagnosis of interstitial, angular, and intrauterine pregnancies in a septate uterus. *J Ultrasound Med.* 2014;33:2031–5.
170. Jiang LY, Wang PH, Lee HY, Chen CY. Diagnosis of interstitial ectopic pregnancy using a three-dimensional high-definition live rendering image. *Taiwan J Obstet Gynecol.* 2015;54:465–6.
171. Arleo EK, DeFilippis EM. Cornual, interstitial, and angular pregnancies: Clarifying the terms and a review of the literature. *Clin Imaging.* 2014;38:763–70.
172. Chen Z, Wang Z, Du M, Liu Z. Artificial intelligence in the assessment of female reproductive function using ultrasound: A review. *J Ultrasound Med.* 2021 Sep 15;41(6):1343–53. doi: [10.1002/jum.15827](https://doi.org/10.1002/jum.15827).

SPERM RECOVERY TECHNIQUES

Clinical Aspects and Laboratory Processing

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In the past three decades, several changes have taken place in clinical andrology. Gradually, empirical treatments are being replaced by techniques of assisted reproduction, i.e. intrauterine insemination, *in vitro* fertilization (IVF), and intracytoplasmic sperm injection (ICSI). In particular, the introduction of ICSI in 1992 [1, 2] has completely changed the clinical approach towards male infertility by offering a novel opportunity for parenthood even to azoospermic men. A single spermatozoon can be injected into an oocyte and result in normal fertilization, embryonic development, and implantation. Not only ejaculated spermatozoa can be used, but epididymal or testicular spermatozoa can also be used for ICSI. Testicular spermatozoa can be retrieved in some patients with non-obstructive azoospermia (NOA) because of the persistence of isolated foci of active spermatogenesis. The first pregnancies using epididymal and testicular spermatozoa in men with obstructive azoospermia (OA) and NOA were published in 1993 and 1995, respectively [3–6]. Surgical retrieval of spermatozoa for ICSI has become a routine technique in clinical andrology. Several techniques are available to retrieve epididymal or testicular spermatozoa. Although there is no real method of choice, some guidelines may be given in order to make the best choice for a specific clinical setting. ICSI has also reinforced the role of non-surgical techniques to retrieve sperm in men suffering from anejaculation.

Azoospermia: What is in a name?

Most azoospermic patients suffer from primary testicular failure (60%) [7, 8]. Because these patients do not show any clinical sign of obstruction, often they are referred to as patients with NOA. However, in a few cases, azoospermia without any obstruction is the result of a hypogonadotropic hypogonadism (i.e. a lack of adequate hormonal stimulation to support spermatogenesis). These patients have an early maturation arrest in spermatogenesis. Treatment with follicle-stimulating hormone (FSH) and human chorionic gonadotropin will restore spermatogenesis, and these patients do not, in the first instance, need assisted reproduction [9]. Thus, these patients are not to be referred to as suffering from NOA and primarily do not need surgical sperm recovery.

Azoospermic patients with primary testicular failure show either a germ cell aplasia (Sertoli cell-only), a maturation arrest, a more severe hypospermatogenesis, or tubular sclerosis and atrophy at their testicular histopathology, whether or not accompanied by focal spermatogenesis in part of them.

Germ cell aplasia may be iatrogenic, as it may result from irradiation or chemotherapy, or it may be congenital because of a genetic disorder such as Klinefelter syndrome or a deletion on the long arm of the Y chromosome. In many cases, however, the cause of germ cell aplasia remains unknown. Many patients with primary testicular dysfunction, however, are now assumed to

have testicular dysgenesis syndrome, a congenital developmental disorder causing spermatogenic failure, maldescent of the testis (cryptorchidism), and eventually hypospadias in more severe forms of this disorder [10]. They also have a higher risk of developing testis carcinoma [10].

Men with NOA may also show maturation arrest at testicular histology. Maturation arrest may be caused by viral orchitis, irradiation, and/or chemotherapy and Yq deletions. Other causes include systemic illness or exposure to gonadotoxins, but here, too, idiopathic maturation arrest is most common. Again, apart from yet-unrevealed genetic causes, testicular dysgenesis syndrome may explain some of these cases.

Fewer men with NOA will show tubular sclerosis and atrophy at their testicular histology. This may be the result of testicular torsion, vascular injury, or infection, but is also a common finding in Klinefelter syndrome patients.

Hypospermatogenesis is a condition in which in all seminiferous tubules all stages of spermatogenesis are present but reduced to a varying degree and, therefore, this condition represents a specific category of NOA as, per definition, surgical sperm retrieval will virtually always be successful [6].

Many studies on assisted reproduction technology (ART) with testicular spermatozoa or spermatids use inadequate definitions, often based on the absence or presence of clinical signs of obstruction. According to World Health Organization (WHO) guidelines for ART, the diagnosis of "NOA" should be made according to the histopathological findings, rather than on the basis of only clinical indicators such as FSH levels or testicular size [8, 11, 12]. Testicular failure is found in a third of normogonadotropic azoospermic men with normally sized testes; on the other hand, small testicular size or elevated FSH does not preclude normal spermatogenesis. Whenever testicular biopsy shows a normal spermatogenesis or a mild hypospermatogenesis, an obstruction of the excretory ducts is present. In a substantial subgroup of these men, however, no clinical signs of obstruction will be present. An accurate distinction between these two types of azoospermia is particularly relevant since spermatozoa can be retrieved in almost all patients with OA and hypospermatogenesis, but only in up to 40%–50% of unselected patients with NOA when no preliminary selection of patients on the basis of histopathology has been performed [13].

Does my patient need surgical sperm recovery?

In patients with OA, fertility can be restored by surgical correction (i.e. vasoepididymostomy, vasovasostomy, or transurethral prostatic resection). When surgery has failed or is not indicated (e.g. patients with congenital bilateral absence of the vas deferens [CBAVD]), surgical sperm recovery procedures are indicated.

Most methods described for surgical sperm recovery are simple techniques. However, in some patients with azoospermia, even these simple techniques are not indicated. When, after appropriate analysis, the diagnosis of azoospermia is made, an appropriate clinical workup is necessary in order to define the exact cause of the azoospermia and to define the best treatment option. If azoospermia is the result of a secondary testicular failure caused by hormonal deficiency, such as hypogonadotropic hypogonadism, then hormone-replacement therapy must be proposed.

Often the diagnosis of azoospermia is made without centrifuging the semen. Centrifugation at $1800 \times g$ for at least five minutes may reveal spermatozoa in the pellet after extended search in micro-droplets, which may be used for ICSI [14]. In 2007, a national survey conducted by Swanton et al. including all 70 IVF units in the United Kingdom, revealed that 91% of these centres routinely performed extended sperm preparation (ESP). In the same communication, the Oxford Fertility Unit presented a series of 87 azoospermic men in which ESP identified sperm in 22% of the cases. This percentage rises to 30%, excluding patients after attempted vasectomy reversal [15]. In cases of NOA, it may therefore be worthwhile to perform centrifugation of at least one ejaculate before embarking on a surgical recovery procedure to retrieve spermatozoa. Only when no spermatozoa are found in the pellet after centrifugation or when only immotile, non-viable spermatozoa are found is surgical sperm recovery indicated in order to avoid performing ICSI with spermatozoa with severe DNA damage.

Anejaculation does not equal azoospermia

Surgical sperm retrieval methods have been proposed as means for obtaining spermatozoa for assisted reproduction in men with anejaculation, i.e. absence of antegrade or retrograde ejaculation. However, given the efficiency of assisted ejaculation in these men, surgical methods are only to be considered when penile vibratory stimulation (PVS) or electroejaculation (EEJ) have failed.

Epididymal or testicular sperm recovery procedures are often proposed to anejaculatory patients because no PVS or EEJ is available [16]. When these first-line recovery methods are unavailable, it is even preferable to refer anejaculatory patients, especially patients with spinal cord injuries (SCI), to specialized services where assisted ejaculation can be performed and semen can be cryopreserved. Vibro- or electro-stimulation are non-invasive techniques that may be performed without any anaesthesia in paraplegic men. EEJ is now a well-established method for procuring sperm from spinal cord-injured men [17]. Since scrotal haematoma or local infections after surgery may take a long time to heal in SCI men, surgical sperm retrieval techniques should be indicated only where these non-invasive techniques fail to produce an ejaculate that may be used for ICSI. Even here, vas deferens aspiration may be preferable because of its low risk of iatrogenic obstruction [18, 19]. The ejaculate, even in cases of oligo-astheno-teratozoospermia, can be cryopreserved for later use. Testicular sperm retrieval must be considered only where primary testicular failure is present in an anejaculatory patient or when techniques of assisted ejaculation have failed to produce an ejaculate that can be used for ICSI. It is preferable in such patients to refrain from epididymal sperm aspiration techniques because of their higher risk of iatrogenic epididymal obstruction. Vas deferens aspiration can also be performed in patients with retrograde anejaculation [19] as an alternative to recovery from post-ejaculatory urine [20].

Psychogenic anejaculation may be encountered unexpectedly during treatments with ARTs. In these patients, assisted ejaculation combined with ART can be an effective approach, achieving acceptable fertilization, pregnancy, and live birth rates [21].

Patients facing IVF treatment can also suffer from erectile dysfunction. If treatment with sildenafil citrate has failed, assisted ejaculation has a role in order to obtain spermatozoa [22]. In some anejaculatory SCI patients, prostatic massage—a simple, alternative, non-invasive method—can be used in order to obtain spermatozoa for ART [23].

Ejaculation induced by PVS and EEJ

Anejaculation may be psychogenic or may result from spinal cord injury or retroperitoneal lymph node dissection. These three causes represent almost 95% of aetiologies. Diabetic neuropathy, multiple sclerosis, Parkinson's disease, and aorto-iliac, colorectal, or bladder neck surgery are less commonly encountered causes. Occasionally, anejaculation is drug-associated: anti-depressive, antipsychotic, and antihypertensive medication may induce anejaculation.

Given the low efficiency of medical treatments for inducing ejaculation in anejaculatory men, PVS (Figure 59.1 and Protocol 2 in the Appendix) and EEJ (Figure 59.2 and Protocol 1 in the Appendix) may be considered as the first-line treatments for anejaculation [24].

PVS is recommended because it is still less invasive and less expensive than EEJ and because semen quality has been reported to be much better after PVS than after EEJ, especially in men with spinal cord injury [25]. PVS can restore ejaculation in half of anejaculatory patients when it is properly used [25].

In case of SCI, each patient scheduled for PVS should undergo an andrological examination and complete neurological examination. PVS needs an intact spinal cord up to the lumbosacral level. In spinal cord-injured men, PVS is less successful in cases of lower cord lesions. When the patient has a transection at T6 or higher, an increase in blood pressure because of autonomic dysreflexia may occur during a PVS procedure. Close monitoring of blood pressure is thus indicated. Whenever acute hypertension



FIGURE 59.1 Penile vibratory stimulation. The vibrator should deliver a high peak-to-peak amplitude of at least 2.5 mm and a frequency of about 100 Hz. The vibrating part is applied to the posterior glans penis and frenulum.



FIGURE 59.2 Electroejaculation. The patient is in lateral decubitus and a stimulatory probe is gently introduced in the rectum with the electrodes facing the prostate.

develops, 10–20 mg nifedipine should be administered sublingually. Alternatively, urapidil 5 mg can be administered intravenously. In spinal cord-injured patients with a history of autonomic dysreflexia, 10 mg nifedipine should be given preventively about 15 minutes before starting PVS.

The SCI patient is instructed to drink 500 mL water containing 600 mg sodium bicarbonate on the morning of the procedure in order to alkalinize the urine. After emptying, the bladder is washed with a buffered sperm preparation medium. About 50 mL of this medium is left in the bladder. The vibrating part of the vibrator is placed on the posterior glans penis and frenulum. The position can be slowly changed in order to find a reactive trigger point. According to the literature, ejaculation can be obtained within 10 seconds up to 45 minutes, but often the procedure is discontinued after 10–15 minutes of trying. Although less frequently than with EEJ, retrograde ejaculation may occur during PVS. Flushing, goose skin, and spasms of the abdominal muscles and legs may indicate ejaculation in SCI patients. In general, spermatozoa can be obtained in approximately 55% of men; however, in spinal cord-injured men with lesions above T11, the retrieval rate reaches 88% [16].

High-amplitude penile vibro-stimulators have become affordable and therefore couples that are infertile because of anejaculation can use PVS at home for attempting pregnancy [26] or to improve semen quality by regular ejaculation. Home-use penile vibro-stimulators may not be indicated in spinal cord-injured men with lesions above T6 because of the risk of autonomic dysreflexia. PVS can also be proposed in men with psychogenic anejaculation without all the preparations that SCI men need. However, there is only anecdotal evidence in the literature about the efficiency of this approach in case of psychogenic anejaculation [27].

EEJ is the treatment of choice if PVS fails. EEJ is a technique initially introduced to obtain spermatozoa from endangered species. In the late 1980s, the technique was introduced successfully in the clinic, too [28]. Patients should receive the same workup and preparation as for PVS.

For EEJ, patients with no spinal cord injury or patients with incomplete spinal cord lesions need general anaesthesia. Sympatholytic agents should not be used during anaesthesia.

As for PVS, SCI men with lesions at T6 or above must be closely monitored for autonomic dysreflexia, and be pre-treated whenever indicated (as mentioned earlier in the chapter).

The patient is placed in lateral decubitus. Because of the risk of rectal burning due to the heating of the EEJ probe, it is recommended to use equipment with a built-in temperature sensor.

The EEJ probe is introduced in the rectum with the electrodes facing the prostate. In spinal cord-injured men, it may be recommended to perform a preliminary digital rectal examination and an anoscopy. A repetitive electrical stimulus of a maximum 5 V is applied for about two to four seconds each stimulus. When no ejaculation, either antegrade or retrograde, is obtained, the voltage may be gradually increased. With a few exceptions, ejaculation occurs at voltages lower than 25 V. During the stimulation, an assistant collects the antegrade fraction. After the procedure, anoscopy is repeated to ensure no rectal lesions occurred. The patient is placed in lithotomy position and the bladder is washed in order to recover any retrograde fraction. In 80%–95% of patients, spermatozoa can be recovered [16, 28]. According to the quality of the specimen obtained, either intrauterine insemination or assisted reproduction by ICSI can be performed. In anejaculatory men, and especially in SCI men, both semen quality and sperm function may be deteriorated because of accumulation of reactive oxygen species, denervation, male accessory gland infection, post-infectious partial obstruction, or post-infectious primary testicular failure. Therefore, the introduction of ICSI has dramatically changed the perspective of patients suffering from anejaculation [16, 29]. Indeed, in combination with ICSI, spinal cord-injured men can father their children who are genetically their own, even when sperm quality is limited. In a small retrospective study, prostatic massage, EEJ, and testicular sperm extraction (TESE) were compared in terms of establishing a pregnancy by ICSI. It was shown that the three techniques resulted in similar pregnancy and live birth rates [23]. A subsequent study showed that spinal cord-injured men who had ICSI with sperm obtained either after PVS or after EEJ had similar take-home baby rates compared to non-spinal cord-injured men [30].

Methods for retrieving epididymal or testicular spermatozoa

If no motile spermatozoa can be obtained from the ejaculate after centrifugation, a sperm retrieval procedure has to be performed. At present, different methods are available to obtain spermatozoa from the vas deferens, epididymis, or testicular mass [19, 31, 32]. The method of choice will depend on the type of azoospermia (non-obstructive or obstructive), surgical skills, and the techniques available in a given setting. If sperm has to be retrieved on an outpatient basis, techniques should be adopted that are compatible with local or loco-regional anaesthesia.

In case of OA, several methods are available. Figure 59.3 shows the algorithm aiming at obtaining spermatozoa with the best maturation status. If OA is expected but either the cause or the site of the obstruction is unknown, a scrotal exploration can be performed. This may not only reveal the cause and site of the obstruction and confirm the diagnosis of OA, but may also provide the possibility of performing reconstructive surgery. If no surgical correction is feasible or indicated because of associated female subfertility, then surgical sperm retrieval for ICSI can be proposed. In couples with a normal female factor, ICSI in combination with a surgical sperm retrieval technique yields similar

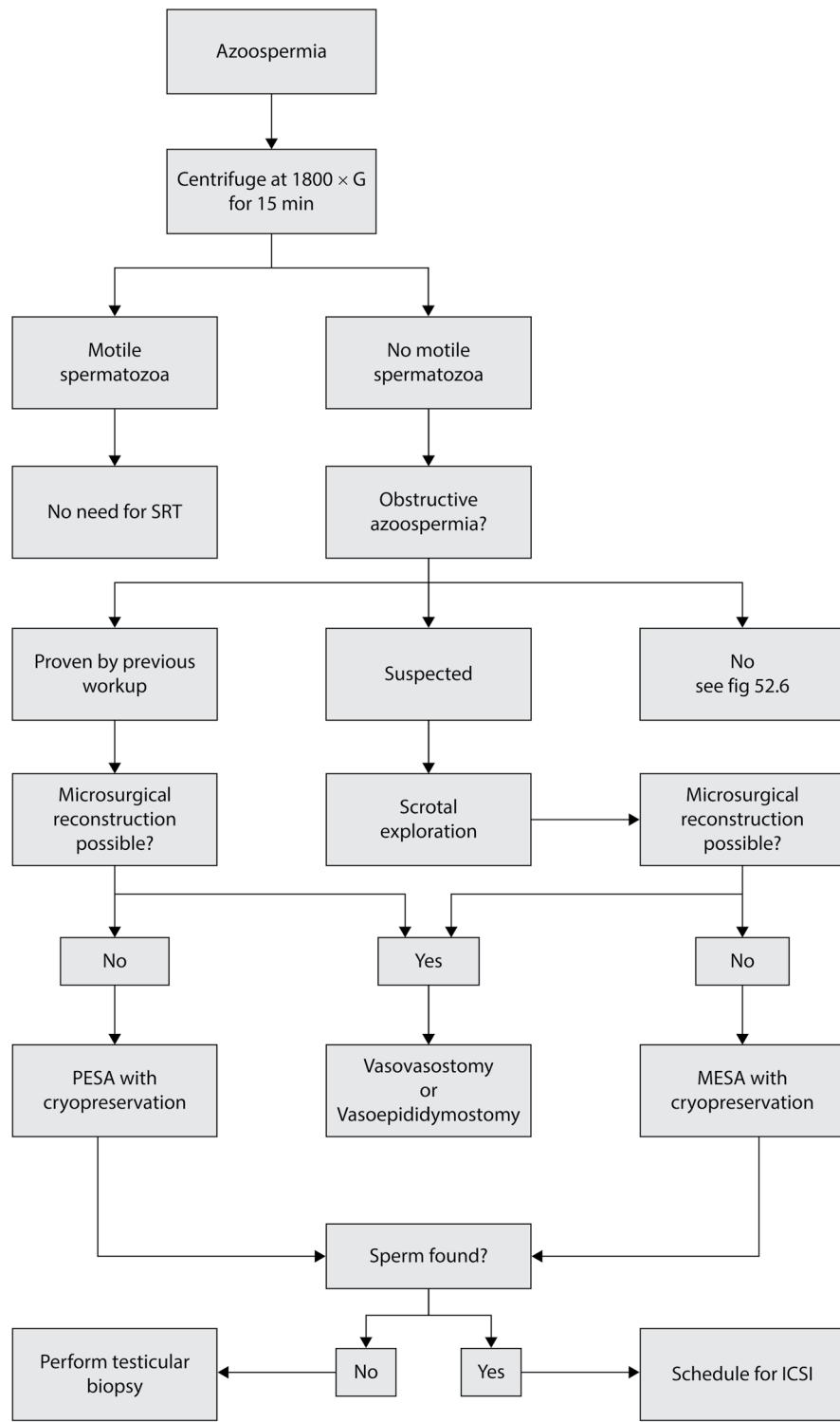


FIGURE 59.3 Treatment algorithm for patients with obstructive azoospermia. Abbreviations: ICSI, intracytoplasmic sperm injection; MESA, micro-surgical epididymal sperm aspiration; PESA, percutaneous epididymal sperm aspiration; SRT, sperm recovery technique.

cumulative delivery rates compared to reconstructive micro-surgery [33].

To retrieve spermatozoa surgically, micro-surgical epididymal sperm aspiration (MESA) can be performed during scrotal exploration. More than 90% sperm retrieval rate can be expected using

this technique (Figure 59.4, Protocol 7 in the Appendix) [34]. The epididymal spermatozoa that are obtained can be easily cryopreserved for later use [35]. A meta-analysis showed no difference in fertilization (relative risk [RR] 1.02; 95% confidence interval [CI] 0.96–1.08) or implantation rates (RR 1.17; 95% CI 0.86–1.59)



FIGURE 59.4 Micro-surgical epididymal sperm aspiration. The epididymis is exposed and epididymal fluid is collected after a micro-incision in a dilated tubule.

after fresh versus frozen-thawed epididymal sperm were used [36]. Although a significantly higher clinical pregnancy rate was observed with the use of fresh epididymal sperm (RR 1.20; 95% CI 1.0–1.42), no difference was found in ongoing pregnancy rates (RR 1.17; 95% CI 0.96–1.43) [36].

If, however, a previous workup has shown that micro-surgical reconstruction is not possible, then a percutaneous epididymal sperm aspiration (PESA) may be preferably performed (Figure 59.5, Protocol 3 in the Appendix). Although there may be some concerns that this blind method may cause epididymal damage and fibrosis, this issue is not important when reconstruction is not possible. When epididymal sperm are to be used for ICSI, spermatozoa with low levels of DNA damage (i.e. motile spermatozoa) are to be obtained in order not to jeopardize the success rate of the coincident ICSI cycle. Epididymal sperm may accumulate DNA damage over time: the study by Ramos et al. reported that about 17% of sperm obtained from the epididymis

by MESA shows DNA damage as demonstrated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay. The DNA damage rate was only 9.3% for sperm recovered from the testis and 6.2% in fresh sperm obtained from sperm donors [37]. However, in the motile fractions of the surgically recovered sperm, the final DNA damage rate was less than 1% and comparable to that of donor sperm [37]. When motile spermatozoa are used, no differences in fertilization rates or live birth rates are observed between epididymal and testicular sperm used for ICSI [36]. PESA has a high sperm retrieval rate, being able to provide motile sperm for ICSI in more than 80% of the patients with OA [38, 39]. In cases of PESA failure, testicular spermatozoa may be obtained, although live birth rates after ICSI using epididymal sperm are higher than after using testis sperm [40].

In men with OA, testicular sperm can be obtained either by fine-needle aspiration (FNA) or by open testicular biopsy of the testis (Figure 59.6, Protocols 4 and 5 in the Appendix). Both methods are similar in terms of outcome in OA, but the number of sperm obtained after open biopsies is much higher [41]. For this reason, testicular biopsy may be preferred whenever cryopreservation is desired. Alternative methods of testicular aspiration have been described yielding higher numbers of spermatozoa [32, 42]. In these aspiration techniques, either needles with a larger diameter are used in order to obtain tissue cylinders or seminiferous tubules are pulled out by micro-forceps after puncturing or incising the tunica albuginea [32]. Compared with FNA, these alternative methods are less patient friendly and need local or loco-regional anaesthesia. Sometimes they even need to be combined with a small incision by a sharp blade in the scrotal skin. Their main advantage is that cryopreservation is easy and efficient because of the higher numbers of sperm obtained.

For men with OA who need surgical sperm recovery for ICSI, both patient and surgeon can decide which approach will be used. When cryopreservation is required, then PESA is the method of choice, followed by TESE whenever the former approach fails to recover motile epididymal sperm. These two techniques yield high numbers of sperm necessary for cryopreservation.

When a minimally invasive technique is preferred (“no-scar technique”), then again PESA is the first-choice method, followed



FIGURE 59.5 Percutaneous epididymal sperm aspiration. The epididymis is palpated and epididymal fluid is collected after a blind percutaneous puncture with a 19- or 21-gauge needle.



FIGURE 59.6 Fine-needle aspiration of the testis. Using a fine 21-gauge butterfly needle filled with a minute volume of sperm preparation medium, the testicular mass is punctured and an aspirate is collected.

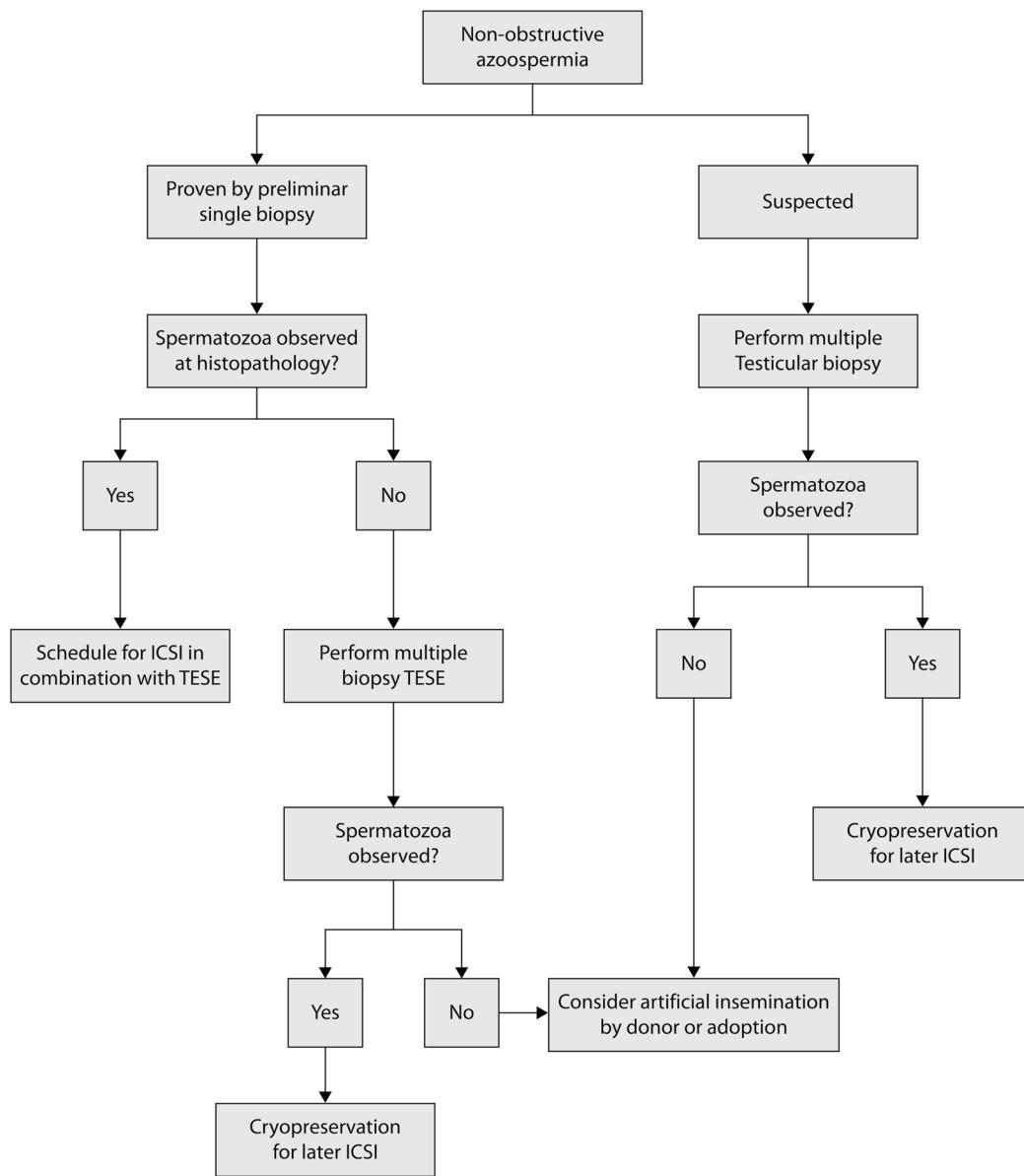


FIGURE 59.7 Treatment algorithm for patients with non-obstructive azoospermia. Abbreviations: ICSI, intracytoplasmic sperm injection; TESE, testicular sperm extraction.

by FNA whenever PESA fails to recover motile spermatozoa. However, with FNA the numbers of spermatozoa are limited, hampering routine cryopreservation.

Figure 59.7 shows our current algorithm for patients with NOA willing to undergo ICSI treatment combining ability to freeze and patient friendliness.

TESE is the appropriate technique for retrieving spermatozoa in NOA with an average sperm retrieval rate of 40%–50% [43, 44]. Although testicular histology remains the best predictor for recovery [45–47], testicular volume and serum FSH are routinely assessed as non-invasive parameters in azoospermic men; however, their predictive power remains limited and is subject to the heterogeneity of the population of NOA patients studied [44, 46–49]. There exist data contradicting inhibin-B has any role as a routine assessment for predicting successful sperm recovery [50–53].

Other non-routine markers (e.g. leptin) have been described as useful in the prediction of sperm recovery after TESE [54]. Since a single parameter has a low sensitivity for predicting TESE outcome, predictive models have been published combining several parameters [54, 55]. At present, it remains, however, to be proven whether such predictive models apply to all azoospermic men [56].

Some authors envision that artificial intelligence could be the solution and that machine-learning methods may be applied to improve prediction models. Zeadna et al. reported that using a gradient-boosted trees algorithm it is possible to achieve high sensitivity (91%) with moderate specificity (51%) in predicting TESE outcome [57]. In their series, the use of the algorithm would have allowed avoidance of surgical intervention in 20 (50% of unsuccessful TESE) of 119 patients. This study has many

limitations; however, machine-learning models and, in the future, deep learning may contribute valuably to clinicians' and patients' decision-making.

Apart from clinical parameters and hormonal tests, Doppler ultrasound of the testis has also been proposed as a method to predict successful recovery. But even such testicular vascularity assessment has a sensitivity not exceeding 50% [58, 59]. In recent years, magnetic resonance (MRI) has been explored as a non-invasive imaging method to predict successful sperm retrieval. Ntorkou et al. reported that some MRI parameters like testicular volume, apparent diffusion coefficient (ADC), and magnetization transfer ratio (MTR) can be helpful in predicting the possibility of obtaining spermatozoa after mTESE [60].

The most invasive predictive strategy for TESE is "testicular mapping." According to an organized pattern, the testis is aspirated in different locations, followed by a cytological examination of these aspirates. Again, the sensitivity of this approach is below 50%. A mapping study by Bettella et al. reported that in 70 patients with a Sertoli cell-only pattern at testicular histology, mapping did not show any sperm; however, during a subsequent TESE procedure, sperm were recovered in 41% of these patients [61, 62].

Although only applicable to a few patients, appropriate Yq deletion testing using carefully selected test markers provides a robust prediction, as no testicular sperm can be recovered in azoospermic men with AZFa and AZFb deletions [63, 64]. The same goes for the rare azoospermic patients suffering from a "de la Chapelle syndrome" or XX (SRY+) male syndrome [65].

The appropriate number of biopsies to be taken remains controversial. Although initially a single testicular biopsy has been proposed as the best approach [66], it is currently recommended to take multiple samples from different sites of the testis, since a patchy distribution of spermatogenesis throughout the testis has been identified [45, 67]. In addition, it has been shown that TESE with multiple biopsies results in a higher chance of finding motile spermatozoa [68]. Care should be taken to take small tissue pieces and to avoid cutting the arterioles as much as possible in order not to cause too much devascularization.

Concerning the best location to perform the biopsy, two small descriptive studies reported opposite results. Hauser et al. [68] found no differences in the sperm retrieval rate between three testicular sites, whereas Witt et al. concluded that the midline portion of the testis enabled the highest retrieval rate [69].

If a preliminary single biopsy has shown focal spermatogenesis with testicular spermatozoa present, the patient and his partner may be scheduled for ICSI with a TESE performed on the day of the oocyte retrieval. Vernaeve et al. reported finding sperm in up to 78% of patients in whom TESE had been previously successful [70].

When a preliminary single biopsy has not shown the presence of testicular spermatozoa, a testicular sperm retrieval procedure with multiple biopsies has to be proposed (Figure 59.8, Protocol 6 in the Appendix) [6, 71]. Ideally, multiple biopsies are only sampled whenever the first biopsy, taken at the testis with the highest volume, does not show spermatozoa after immediate search in the wet preparation. Because multiple biopsies may lead to extensive fibrosis and devascularization [72, 73], multiple excisional biopsies may be taken under an operating microscope at $\times 40$ and $\times 80$ magnification [74]. This micro-surgical approach aims at sampling the more distended tubules in order to limit testicular damage. Micro-TESE (Figure 59.9) may theoretically be very useful in cases of Sertoli cell-only syndrome with focal spermatogenesis,



FIGURE 59.8 Multiple testicular sampling by open excisional testicular biopsy (testicular sperm extraction). Small tissue specimens are taken from the testicular mass while avoiding vascular injuries when incising the tunica albuginea.

but less useful in cases with maturation arrest where there is generally no difference in diameter of tubules with or without focal spermatogenesis. The technique is more time-consuming than conventional TESE, needs an operating microscope or high-magnification operating loupes, and may be influenced by the surgeon's case volume [75]. Some authors recommend micro-TESE as a salvage technique when TESE is negative in NOA patients, reaching a sperm retrieval rate of 46.5% [76]. A recent systematic review and meta-analysis comparing sperm recovery rate (SRR) in conventional TESE versus micro-TESE (mTESE) analysed 15 studies with a total of 1890 patients. The authors concluded that mTESE was 1.5 times more likely to retrieve sperm than conventional TESE (95% CI 1.4–1.6) [77]. Nonetheless, studies included in this meta-analysis were not randomized and showed high heterogeneity in both patient population and laboratory processing of samples, and thus definitive conclusions cannot be drawn. A second meta-analysis concluded that in patients with



FIGURE 59.9 Testicular sampling by microscope-guided testicular sperm extraction (TESE) or micro-TESE. Under magnification, a dilated seminiferous tubule is excised using microscissors.

(incomplete) Sertoli cell-only syndrome, mTESE may have a benefit over TESE, but put a critical warning to the fact that their data set only covered small pseudo-randomized studies [78]. Not surprisingly, a recent large retrospective study reported retrieval rates after mTESE that were similar to those from large retrospective studies on conventional random TESE [79, 80]. Finally, the most recent meta-analysis, with rebuttals, ended the controversy by concluding that the current evidence cannot support any superiority of mTESE versus cTESE in patients with NOA [81–83].

When sperm are found, the samples may be frozen for later use with ICSI. If only a few spermatozoa are available or only a tiny amount of tissue is cryopreserved with only a few spermatozoa observed, we ask the patient to be on standby on the day of oocyte retrieval in case no spermatozoa can be observed after thawing. In the literature, data on the use of cryopreserved sperm from NOA patients is scarce. In the study by Verheyen et al., the frozen-thawed suspensions could not be used in 20 out of 97 cycles (20.6%), despite extensive search for motile sperm. However, a backup fresh retrieval was successfully carried out in 14 cycles. Donor semen backup should also be discussed prior to treatment [84]. One meta-analysis that evaluated the impact of fresh versus cryopreserved sperm in a large series of NOA men (574 ICSI cycles) showed no statistical difference in fertilization and clinical pregnancy rates [85].

A special subgroup of NOA is Klinefelter syndrome patients, accounting for 11% of men with azoospermia. Again, in almost half of these patients, spermatozoa may be recovered for ICSI, and pregnancies have been obtained after ICSI with testicular spermatozoa from 47,XXY non-mosaic Klinefelter syndrome [86–89]. Also here, current evidence does not indicate that mTESE is superior to conventional multiple TESE [89]. Age may represent an important parameter to predict sperm recovery in this group of patients, as shown by Okada et al., with a cut-off value of 35 years [90]; however, this finding is not corroborated by compiled data [89]. Whether pre-implantation genetic diagnosis should be performed because of the risk of aneuploidy in the embryos obtained in these patients is another important issue. Staessens and colleagues found that 46% of the embryos were chromosomally abnormal, with a significant increase in sex and autosomal chromosome abnormalities, although without an increased specific risk for 47,XXY [90, 91]. This finding is in accordance with the hypothesis that Klinefelter syndrome men in which testicular spermatozoa can be obtained produce these spermatozoa from 46,XY testicular stem cells [92, 93].

The ultimate goal to perform SSR in NOA men is fathering a genetically own child. Despite the fact that sperm retrieval rates in NOA men, including Klinefelter patients, are between 40% and 50% in general [81, 89], eventually the delivery rate after SSR in combination with the outcome of ICSI once spermatozoa are recovered in men with NOA is lower than expected, i.e. around 15% per started TESE procedure [80, 81], with similar outcomes in Klinefelter patients embarking for this combination [89, 94].

Oncological patients are another subgroup at risk for NOA because of germ cell loss. Patients undergoing potentially sterilizing chemotherapy must bank their semen before starting any treatment [95, 96]. However, they may be azoospermic at the time of cancer diagnosis because of spermatogenic depression due to factors related to the malignancy. Yet these patients may be offered sperm recovery and banking before starting chemotherapy by vasal or epididymal sperm aspiration during orchectomy [97] or TESE (onco-TESE) [98, 99]. Whenever sperm was not banked before starting chemotherapy, some patients with

post-chemotherapy azoospermia may still benefit from TESE [100, 101].

In order to improve retrieval rates in NOA, pre-surgical medical treatment has been proposed, attempting to improve spermatogenesis and eventually sperm recovery [102, 103]. Administration of oestrogen receptor modulators such as clomiphene citrate or tamoxifen citrate and aromatase inhibitors primarily focuses on the enhancement of intra-testicular testosterone levels and FSH production, but also increases plasmatic oestrogen levels as well as testosterone production.

Treatment of infertile males with aromatase inhibitors like testolactone, anastrozole, and letrozole has been associated with increased sperm production and return of sperm to the ejaculate in men with NOA in small clinical trials and case reports [104–106]. Some authors postulate that the use of aromatase inhibitors could enhance the sperm retrieval rate in a selected group of patients with a low testosterone over oestradiol (T/E2) ratio (<10), such as Klinefelter syndrome patients [105]. Unfortunately, neither large cohort nor randomized controlled trials on either aromatase inhibitors or clomiphene citrate are available, hence the off-label use of this medical pre-treatment for NOA patients with low serum testosterone and abnormal T/E2 ratios should be carefully discussed before undergoing TESE. Moreover, a more recent larger study could not corroborate the findings of earlier studies and case reports [107].

The main complications of testicular retrieval techniques, especially when taking multiple biopsies, are haematoma, infection, fibrosis, and testicular atrophy [70, 108, 109].

Less invasive methods have been proposed in order to obtain testicular spermatozoa from patients with NOA, i.e. testicular aspiration. The main advantages of this technique are simplicity, low cost, being minimally invasive, and that it produces less post-operative pain compared to TESE under local anaesthesia [31]. However, multiple prospective studies have shown a lower recovery rate than with excisional biopsies [31, 110–113]. In patients with a history of cryptorchidism who have a higher risk of developing a testicular cancer from carcinoma *in situ* cells, an excisional biopsy must be performed in order to check for carcinoma *in situ* [114].

As long as well-powered randomized trials are missing, controversy will continue to exist regarding which sperm retrieval technique should be preferred in NOA patients to guarantee the best chances of retrieving spermatozoa. While many studies (unfortunately poorly designed) focus on the surgical aspects of retrieving sperm, only a few studies address retrieval from testicular samples in the IVF laboratory. In many IVF laboratories, sperm retrieval is limited to microscopic observation of the wet preparation. However, the retrieval may be facilitated in the laboratory by extended search [115], use of erythrocyte-lysing buffer [116, 117], and enzymatic digestion [118–120]. Some authors have reported that scheduling the testicular recovery procedure one day before the ovum pickup [121] or the use of motility stimulants, e.g. pentoxifylline, may facilitate the retrieval of motile spermatozoa from the tissue [122–124].

Processing testicular sperm in the lab

Once the harvested testicular tissue enters the lab, different methods are available to process the sample for ICSI or cryopreservation. Each technique should be optimized to achieve the highest likelihood of detecting sperm while protecting the spermatozoa from unnecessary mechanical, enzymatic, oxidative, and thermal stress [125].

The first step in processing a testicular biopsy is rinsing (at 37°C) the tissue in sperm wash media or Hepes buffered media to rinse away contaminants like erythrocytes and fibroblasts. Afterwards, a mechanical disruption is performed to open the seminiferous tubules and disperse the spermatozoa that are present in the tubules. In 1995, Verheyen and colleagues [126] compared different mechanical methods to prepare testicular biopsies and found that mincing—compared to other mechanical methods like rough shredding, vortexing, and crushing with an electric potter—resulted in the highest yield of total motile spermatozoa and percentage of spermatozoa with normal morphology. Mincing is usually performed with sterile scissors or 18G needles. Finer homogenization can be performed by repeated aspiration and passage of the testis tissue through a fine-gauge hypodermic or angiocatheter needle [127]. Mechanical dispersion and passage through a 24-gauge angiocatheter needle has been associated with a 470% improvement in the sperm retrieval rates compared to mincing alone [128]. Mechanical processing is a fast technique to recover sperm, but introduces the risk of cellular injury through shearing force. These mincing steps are followed by a microscopic evaluation using 400× magnification to identify (motile) spermatozoa. In case no spermatozoa are found, the testicular biopsy sample can be purified of erythrocytes using erythrocyte lysing buffer or density gradient centrifugation. After discarding the supernatant, the remaining pellet should be resuspended in culture medium and re-examined [129, 130].

Besides the mechanical release of spermatozoa, non-mechanical processing techniques, such as enzymatic digestion, also exist. Especially as the mincing process is not always able to release all spermatozoa from the tubules, the mincing technique is often combined with enzymatic digestion [130–132]. Enzymatic digestion was optimized by Crabbé and colleagues [133] in 1997 and showed that collagenase type IV appeared to be the most efficient in isolating spermatozoa from testicular tissue. The process of enzymatic digestion is performed by incubating the testicular tissue with type IV collagenase and DNase for up to two hours at 37°C and mixing the solution regularly to increase the amount of tissue exposed to the collagenase. The digested tissue is centrifuged and washed with sperm media or Hepes buffered media [134]. Wober et al. [130] recently described their technique of enzymatic digestion and density gradient centrifugation to further improve testicular sperm purity. A retrospective multicentre comparison of mechanical versus enzymatic preparations of 839 ICSI cycles using testicular sperm revealed a significantly higher percentage of cycles with motile sperm following mechanical preparation ($p = 0.03$) [135]. Despite the potential of collagenase to damage the sperm cell membrane, a 24-hour vitality study showed no difference between enzyme-exposed sperm and untreated sperm obtained from men undergoing orchietomy as part of androgen deprivation therapy or from residual fragments of testis tissue following an ICSI treatment [133]. Of note, in case the testicular biopsy sample is retrieved as part of a diagnostic treatment, enzymatic digestion can be skipped, as the cryopreservation process itself will also partially “digest” the tissue, leading to the release of spermatozoa upon thawing.

Cryopreservation and thawing of testicular sperm

Cryopreservation of testicular sperm is of special importance for patients with male factor infertility, be it patients with testicular sperm extraction or patients with severe oligozoospermia. Even

though sperm survival rates post-thaw still fluctuate around 50% [136], cryopreservation of sperm allows couples to make informed decisions on their future treatments (e.g. switch to donor sperm). Also, cryopreservation of sperm reduces the need for repeated sperm retrieval procedures, as testicular injury can be incurred with each extraction procedure [125]. To increase success rates for NOA patients, continuous efforts are made in the cryopreservation of (testicular) sperm, with more recent focus on the cryopreservation of single sperm [137].

The impact of slow cooling on spermatozoa was originally described by Sawada et al. [138] in 1967 and later corroborated by Leibo [139] with demonstration of extracellular ice formation, plasma membrane damage, and cell death. Slow thaw rates can also create additional destructive forces by allowing maximal growth of ice crystals [140]. Furthermore, reactive oxygen species formation during the freeze-thaw cycle pose a threat to the diverse cellular compartments and genetic integrity of the sperm. Regulation of cooling and warming rates of the freeze-thaw cycle and the use of cryoprotectants and semen extenders therefore represent means of preventing phase transition’s lethal cellular injury and ultimately improve sperm cryo-survival. In addition to optimizing osmotic pressures and preserving membrane integrity, semen extenders provide an alternative energy source for sperm metabolism and reduce the breakdown of intracellular sperm phospholipid [141].

Successful recovery of spermatozoa following a freeze-thaw cycle was first demonstrated in 1995 by Craft and Tsirigotis [142], followed by successful fertilization of oocytes with cryopreserved testicular sperm by Romero and colleagues [143], though no pregnancies were achieved. Shortly thereafter, Oates et al. [127] published a case series of 10 couples with NOA males and showed fertilization rates of 48% with frozen-thawed testicular sperm and a pregnancy rate of 11%. A meta-analysis, comparing fresh and cryopreserved testicular sperm in NOA patients, was unable to show differences in fertilization and pregnancy rates [144].

Current testicular sperm cryopreservation techniques include slow freezing (SF), rapid freezing (RF), and ultra-rapid freezing (URF) protocols. In the three protocols, testicular spermatozoa are dropwise mixed with commonly used cryo-protectants such as egg yolk, which supplement lipids necessary for membrane fluidity and stability of the acrosin/proacrosin enzyme system, and intracellular glycerol, which penetrates the cell to replace most intracellular water and lowers the intracellular freezing point [145–147]. Comparisons of SF and RF protocols have demonstrated superior post-thaw motility and cryo-survival with RF, but no differences in post-thaw sperm morphology and sperm DNA integrity [148]. A recent comparison of the URF and SF protocols demonstrated a statistically significant reduction in post-thaw sperm motility after one month of cryopreservation with both protocols; however, the difference between the protocols was not significant ($p > 0.05$) [149].

The impact of various storage and thawing temperatures has been evaluated to determine the optimal cryopreservation temperatures. Comparisons of storage at -70°C to the conventional storage temperature at -196°C demonstrated superiority of -196°C in post-thaw sperm motility at seven days and three months after the initial freezing [150]. Thaw protocols regulate the ascent of specimen temperature in order to prevent rapid and dramatic changes in cell volume and cell injury associated with shifts of water into the cell and exchange with glycerol [145]. Three thawing protocols include thawing at room temperature for 15 minutes, combination of thawing at room temperature for

10 minutes and 10 minutes in a 37°C water bath, or thawing in a 37°C water bath for 10–20 minutes. Comparison of thawing protocols of using the combined 10-minute room temperature thaw and 37°C water bath versus placing the sample in a 37°C water bath for 20 minutes have shown a higher percentage of fast linear movement and viability with less acrosomal damage associated with the 37°C water bath [151].

A major issue arising from the cryopreservation of testicular sperm in large volumes (300–500 µl straws), is the labour-intensive search for sperm post-thaw. This urges the need to optimize protocols for single sperm cryopreservation. This technique allows the cryopreservation of a single or few sperm cells on a single device. Its importance is clear after testicular biopsy, but also in patients with severe oligozoospermia, necrozoospermia, and even patients with fertility preservation. A multitude of different biological and non-biological carriers have been tested, like empty zona pellucidae or *Volvox globator* spheres, polymerized alginic acid capsules, mini-straws, 5-mm copper loops, calcium alginate beads, hyaluronan microcapsules, microdroplets, Cryolock, agarose gel microspheres. The results of these devices were recently reviewed by Liu and Li [136]. An initial systematic review in 2009 showed a recovery rate of 79.5% (range 59%–100%), a cryo-survival rate of 46.5% (range 8%–85%), and a fertilization rate of 42.5% (range 18%–67%) for all carriers [152]. More recently, 13 carriers were evaluated in a systematic review by Huang and colleagues [153] and showed increased recovery rates of 92% (95% CI: 87%–96%) with tremendous increases in survival (76% [95% CI: 69%–83%]) and fertilization rates (63% [95% CI: 58%–67%]). Delivery rates of 40% (95% CI: 12%–71%) were reported.

Though the latest results for single sperm cryopreservation seem promising, in-depth studies are still required to achieve optimal freezing results for this special patient population.

A successful testicular sperm recovery: What is next?

In earlier reports, pregnancy rates after ICSI using testicular spermatozoa were comparable to those obtained after ICSI using epididymal spermatozoa patients with normal spermatogenesis [36, 156]. However, more recently, results with testicular sperm were found to be inferior to epididymal sperm [40]. Unfortunately, all these reports are based on retrospective case series and thus high-quality evidence in favour of the use of epididymal sperm in OA men is lacking.

In NOA men, ICSI with testicular sperm results in lower fertilization and embryo development compared with either the sperm of OA individuals or the ejaculated sperm of non-azoospermic men [157, 158]. The reasons for this finding remain unclear, but higher aneuploidy rates in men with NOA have been suggested [159], and pre-implantation genetic testing (PGT-A) was eventually proposed as a way to improve embryo selection in azoospermic men because of a higher frequency of aneuploid and mosaic embryos [160, 161]. However, whether PGT-A via comprehensive chromosome screening has any benefit for ICSI in NOA men yet remains to be proven [162, 163].

When comparing reports on ICSI in NOA men, significant differences do exist between various reports, mainly because of differences in patient selection, the sample size of the study, and the definition of NOA [80]. Typically, ICSI success rates in NOA patients are reported in different patient populations in which

eventually testicular spermatozoa were invariably obtained [158, 164]. Only a few studies provide data on cumulative delivery or pregnancy rates after ICSI; however, again, these studies only include patients with successful sperm retrieval and thus overestimate pregnancy and live birth rates [165–167].

Currently, only one retrospective cohort study reports on a longitudinal follow-up of unselected NOA men undergoing TESE and eventually ICSI, concluding that while one out of four couples undergoing ICSI will have a live birth, eventually only one out of seven men undergoing TESE will father a child that is genetically their own [80].

Few data are available about the pregnancy outcomes and the neonatal data of children born after ICSI with surgically retrieved sperm in patients with azoospermia, and often such studies do not discriminate between either the source (epididymal vs testicular) or the testicular function (obstructive vs non-obstructive) [168, 169].

So far, based on the few studies available, there is no indication that using either epididymal or testicular sperm from azoospermic men is associated with an increased risk of neonatal health problems, congenital malformation, or aneuploidy in comparison to children born after ICSI with ejaculated sperm [168, 170].

Based on small sample sizes, these data have not shown any difference between pregnancies after the use of testicular sperm from NOA men compared to OA men [171, 172].

Patients should thus be counselled that treating sterility because of OA is a successful approach, while ICSI for NOA has many limitations: firstly, there are limitations in the chances to recover testicular spermatozoa; and secondly, there are limitations in the outcomes after ICSI itself. ICSI with surgically retrieved sperm has been reported to be similar to ICSI with ejaculated sperm in terms of safety; however, there is an urgent need for longer follow-up with adequately powered and prospective cohort studies.

Appendices

Protocol 1: EEJ

Indication

Anejaculation refractory to PVS.

Patient preparation

In spinal cord-injured men, a preliminary microbiological examination of the urine has to be performed. No rectal preparation (such as klysma). Fluid intake restricted to 500 mL in the 12 hours preceding the procedure.

The patient has to empty the bladder before EEJ. In spinal cord-injured men with lesions at T6 or higher, monitoring of blood pressure is mandatory. Sublingual nifedipine 10–20 mg may be given for preventing autonomic dysreflexia-related hypertension. Urapidil 5 mg IV is an alternative for nifedipine.

Patient wears a top only. He is placed in lithotomy position. Penile region cleansed with antiseptic solution (e.g. HAC, Zeneca: hospital antiseptic concentrate—contains chlorhexidine).

The EEJ procedure

The tip of a Nelaton bladder catheter is dipped into sterile liquid mineral oil as used in IVF. After instillation of 10 mL of sperm preparation medium into the urethra, the catheter is gently introduced into the bladder. The bladder is emptied and the urinary pH is measured. The bladder is then washed with 200 mL medium. After emptying, 50 mL of the medium is left in the bladder for

collecting retrograde-ejaculated sperm. The patient is put into lateral decubitus. In spinal cord-injured men, an assistant should control leg spasm during the procedure.

Electro-stimulation is performed using equipment with a built-in temperature sensor. After digital rectal examination and anoscopy, a standard probe is gently inserted into the rectum. Care is taken to orient the electrodes anteriorly. Electro-stimulations are repeated, each stimulation lasting for two to four seconds. Baseline voltage should be 5 V and voltage can be increased or maintained according to the patient's reaction. In case of acute hypertension in patients with spinal cord lesions at T6 or higher, the procedure must be discontinued until blood pressure is again under control.

An assistant collects the antegrade fraction in a sterile container holding buffered sperm washing medium. The pendulous and bulbar urethra are continuously massaged by the assistant during the procedure. With the aid of a 1-mL syringe, ejaculated drops are flushed into the container. When no antegrade ejaculation is observed, indirect signs such as spasms of the lower abdominal muscles and legs and the appearance of goose bumps may indicate (retrograde) ejaculation. When ejaculation discontinues, the probe is removed and anoscopy is performed again to check for rectal lesions.

Then the patient is put again in lithotomy position. The bladder is re-catheterized and the bladder is emptied into a sterile container in order to collect any retrograde fraction. The bladder is flushed with 100 mL of medium until the flushing medium remains clear.

The collected fractions are transported to the andrology laboratory for identification of spermatozoa and further preparation. Centrifugation of the retrograde suspension may be necessary or open biopsy under local anaesthetic should be performed.

Dressing after

Disposable underpants.

Patient care post-operation

None.

Requirements

- A runner
- Two assistants
- Seager Model 14 Electroejaculator (Dalzell Medical System, The Plains, VA, USA)
- Anoscope
- Manual manometer
- Nelaton catheter ch 14 (Cat.nr. 110)
- pH indicator strip (Merck, Germany)
- Mineral oil (Sigma-Aldrich, Darmstadt, Germany)
- Cleaning solution (3.5% HAC)
- Syringe 50 cc (BS-50 ES Terumo)
- Syringe Norm-Ject Cook 1 mL (K-ATS-1000)
- 100 mL modified Earle's balanced salt solution with 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), 0.4 Heparin Novo, and 2.25% human serum albumin
- Gauze squares 10 × 10

Protocol 2: PVS

Indication

Anejaculation.

Patient preparation

As for EEJ.

The PVS procedure

Patient empties his bladder before PVS and the urinary pH is measured. PVS is performed using high-amplitude equipment.

The antegrade fraction is collected into a sterile container holding buffered sperm washing medium. When no ejaculation occurs after five minutes, PVS is discontinued.

Then the patient is put again in lithotomy position. When no antegrade ejaculation is observed, but indirect signs are present (e.g. goose bumps and muscular spasms), the bladder is catheterized and emptied into a sterile container in order to collect any retrograde fraction (see above). The collected specimens are transported to the andrology laboratory for identification of spermatozoa and further preparation.

When PVS fails to induce ejaculation, EEJ has to be performed.

Patient care post-operation

None.

Requirements

- Ferticare Personal Vibrostimulator (Multicept ApS, Denmark)
- Manual sphygmomanometer
- Ph indicator strip (Merck, Germany)
- Cleaning solution (3.5% HAC)
- Syringe Norm-Ject Cook 1 mL (K-ATS-1000)
- 50 mL modified Earle's balanced salt solution with HEPES, 0.4 Heparin Novo, and 2.25% human serum albumin

Protocol 3: PESA

Indication

All cases of OA with normal spermatogenesis, such as congenital absence of the vas deferens and failed vasectomy reversal (CBAVD patients: read the section "Caveat" in Protocol 7).

Patient preparation

The man is given hibitane soap to wash the area the night before and the morning of the operation. He is also asked to shave the area.

Meperidine hydrochloride 1 mg/kg intramuscularly and midazolam 2.5 mg intramuscularly may be given.

Patient has to empty the bladder before surgery.

Patient is fully draped with the operation site obscured to the patient. Patient wears a top only. Operation site cleansed with antiseptic solution (e.g. HAC, Zeneca—contains chlorhexidine). Penis is held up out of the way with a swab fixed underneath the drape. A drape with a small hole of 5 cm in diameter in the middle covers the operation site. The testes are gently pulled through to be in the field of the procedure. Local anaesthetic—1–2 mL of 2% lidocaine (without epinephrine)—is injected in the spermatic cord in order to obtain loco-regional anaesthesia and into the scrotal skin.

The PESA procedure

A 19- or 21-gauge needle is used. Attached is a 10-mL syringe. The epididymis is held firmly between two fingers of one hand and the needle is inserted with the other hand perpendicular to the epididymis. The needle is inserted into the epididymal mass and then gently withdrawn under slight suction. Care is taken not to move the needle in order to minimize contamination with blood and prevent epididymal damage. The embryologist/nurse brings a 1.5-mL Eppendorf micro-test tube filled with culture medium. The needle is placed in the micro-test tube and rinsed several times with the medium. The micro-test tube is then passed to the

embryologist for identification of spermatozoa. Centrifugation of the suspension may be necessary. The procedure can be repeated if not enough sperm are retrieved. However, if after two aspirations there is no success, then an aspiration of the testis or open biopsy under local anaesthetic should be performed.

Dressing after

Gauze squares and disposable underpants.

Patient care post operation

The man is told that there may be some pain, but it should be minimal. Acetaminophen (paracetamol) can be taken. If more is required, then he should contact the clinic.

Requirements

- A runner
- Drape with central hole
- Non-iodine cleaning solution
- Syringe 10 cc (BS-10 ES Terumo)
- Micro-test tube 1.5 mL (Eppendorf 3810) (to be washed and sterilized first)
- Medium with modified Earle's balanced salt solution, HEPES, 0.4 Heparin Novo, and 2.25% human serum albumin
- Gauze squares 10 × 10 (35813 Hartmann)

Protocol 4: FNA of testis for sperm retrieval

Indication

All cases of OA with normal spermatogenesis, such as congenital absence of the vas deferens and failed vasectomy reversal (CBAVD patients: read the section "Caveat" in Protocol 7).

Patient preparation

As for PESA (see Protocol 3).

The FNA procedure

A 21-gauge 3/4-inch butterfly needle is used; attached is a 20-mL syringe. A small amount of culture medium is drawn up into the tubing and the majority expelled until only about 1–2 mm is left in the butterfly tubing. There may be no air in the fluid. The butterfly needle is inserted perpendicular to the testis and a little away from the site of insertion of the needle used to inject the local anaesthetic, as there is usually some blood at that site. The testis is held firmly in one hand and the butterfly needle is inserted with the other. Care is taken not to move the butterfly needle in order to minimize contamination with blood and prevent testicular damage. The patient may feel some pain only when the needle enters the tunica. The operator or assistant now "pumps" 5–10 times on the 20-mL syringe in order to generate suction to aspirate sperm. It is important to keep a slight negative pressure in order to make sure the aspirate is not pushed back into the testis. This is done by ensuring the plunger does not return all the way to the end. The butterfly needle tubing is then occluded near the needle and the butterfly needle subsequently removed with a smooth, sharp movement in order to minimize tissue trauma and contamination with blood. Occluding the tubing prevents aspirating blood from reaching the skin surface. With the tubing still occluded, the 20-mL syringe (must have rubber stop that may never be in contact with the medium) is removed and a 1-mL syringe with the plunger partially withdrawn is attached. Otherwise, the 20-mL syringe may be used.

The embryologist/nurse brings a dish with nine droplets of culture medium placed on it (one central droplet surrounded by eight droplets). The butterfly needle is placed in a droplet of culture medium and the butterfly needle tubing released, thereby

removing the negative pressure. A small amount of the aspirate and the culture medium in the butterfly needle is then injected into each droplet in turn. Usually about three to five droplets will be used in this way. Fractionating the aspirate containing red blood cells will improve subsequent visualization under the microscope. The dish is then passed to the embryologist for identification of spermatozoa. The procedure can be repeated if not enough sperm are retrieved initially. However, if after three aspirations there is no success, then an open biopsy under local anaesthetic should be performed.

Dressing after

As for PESA (see Protocol 3).

Patient care post-operation

As for PESA (see Protocol 3).

Requirements

- A runner
- Drape with central hole
- Non-iodine cleaning solution
- Syringe 20 cc (BS-20 ES Terumo)
- Surflo Winged Infusionset
- CE 0197 21-gauge × ¾-inch (SV-21BL Terumo)
- Flushed with medium
- Modified Earle's balanced salt solution with HEPES, 0.4 Heparin Novo, and 2.25% human serum albumin
- Syringe 1 cc (Air-Tite K-ATS-1000 Cook)
- Gauze squares 10 × 10 (35813 Hartmann)
- To transport sperm
- Tissue culture dishes (3200 Falcon Becton Dickinson)
- With droplets of medium (modified Earle's balanced salt solution with HEPES, 0.4 Heparin Novo, and 2.25% human serum albumin)

Protocol 5: Open testicular biopsy under local anaesthesia

Indication

Patients with OA with normal spermatogenesis who wish to have testicular sperm cryopreserved (CBAVD patients: read the section "Caveat" in Protocol 7).

Patient preparation

As for PESA (see Protocol 3).

Procedure

Approximately 5 mL lidocaine (2%) is injected into the skin and the underlying layers up to the tunica albuginea. The testis is fixed in the left hand and a 1–2-cm incision is then made into the scrotum and down through the tissue made oedematous by the lignocaine to the tunica. The testis must remain fixed in order not to lose the alignment of the scrotal incision with the incision into the tunica. With the sharp point of the blade, the tunica is opened and the incision slightly extended. Under gentle pressure with the left hand, testicular tissue will protrude through the incision. By the use of a curved pair of Mayo scissors, a small sample is excised and placed into a Petri dish filled with sperm preparation medium (e.g. Earle's). Selective haemostasis with diathermy is performed since intra-testicular bleeding may cause discomfort and fibrosis.

The testicular tissue is rinsed in the medium and then placed into another Petri dish filled with medium. After haemostasis, the tunica is closed with 3/0 Vicryl sutures. The skin is closed

with interrupted 3/0 Vicryl sutures. A clean gauze swab covers the suture site and disposable underpants are given for support.

Patient care post-operation

As for PESA (see Protocol 3).

The patient is told that the sutures will dissolve. There is increased risk of haematoma. The patient should report undue bruising or pain that is not alleviated with paracetamol.

Requirements

- An assistant and a runner
- Monopolar pencil with needle and cord (E 2502 Valleylab)
- Tubeholder (1x) (708130 Mölnlycke)
- To fix cords on drape (pencilcord off foot end)
- Needleholder Mayo-Hegar (20-642-16 Martin)
- Straight Mayo scissors (11-180-15 Martin)
- Adlerkreutz pincer (12-366-15 Martin)
- Allis forceps (30-134-15 Martin)
- Kryle forceps (13-341-14 Martin)
- Micro-Adson pincer (2x) (12-404-12 Martin)
- Micro-Adson pincer (2x) (12-406-12 Martin)
- Adson pincer (31-09770 Leibinger)
- Adson pincer (31-09772 Leibinger)
- Metzenbaum scissors (11-264-15 Martin)
- Metzenbaum scissors (11-939-14 Martin)
- Knife handle with blades nr 15 (0505 Swann-Morton)
- Swabs 10 × 10 (35813 Hartmann)
- Vicryl 3/0 (JV 497 Ethicon Johnson/Johnson)
- Tissue culture dishes (2x) (3102 Falcon Becton Dickinson)
- With medium (modified Earle's balanced salt solution with HEPES, 0.4 Heparin Novo, and 2.25% human serum albumin)
- Local anaesthesia
- Syringe 20 cc CE 0197 (BS-20 ES Terumo)
- Needle 18 gauge (NN 1838 S Terumo)
- Needle 26 gauge (NN 2613 R Terumo)
- Xylocaine 2% (Astra Pharmaceuticals)

Protocol 6: Testicular biopsy under general anaesthesia

Indication

All cases of NOA (primary testicular failure). When testicular biopsy is performed in such patients, a preliminary screening for deletions of the Yq region of the Y chromosome is preferable in the male partner, since deletions may be found in about 5%–10% of patients with unexplained primary testicular failure. Before undertaking the procedure, it is important to identify the best testis to explore. This is done by reading any previous histology reports and feeling the testis for size and consistency. If the testis is high or retracted, then the chance of retrieving spermatozoa is lower.

Patient preparation

As for PESA (see Protocol 3).

Procedure

Biopsies taken at random

As for under local anaesthetic (see Protocol 4). The main difference is that a larger scrotal incision is made and the testis is delivered.

If no sperm are observed in the wet preparation, multiple small incisions can be made and biopsies taken accordingly. The

incisions must avoid the arterial blood supply. The contralateral testis may be explored as well.

Biopsies taken with operating microscope (micro-TESE)

After scrototomy, the tunica albuginea is opened longitudinally with the sharp point of the blade, avoiding the arterial blood supply. Then the testicular pulpa containing the tubuli seminiferi is exposed to a 40–80× magnification using an operating microscope. Care is taken to keep the tubuli wet by a constant drip of saline. Distended tubules are spotted and sampled by microscissors, avoiding the arterial blood supply.

The tiny samples are placed into a Petri dish filled with sperm preparation medium (e.g. Earle's). The testicular samples are rinsed in the medium and then placed into another Petri dish filled with medium. After controlling haemostasis, the tunica is closed with a continuous 7/0 Ethilon suture. The skin is closed with interrupted 3/0 Vicryl sutures. A clean gauze swab covers the suture site and disposable underpants are given for support.

Patient care post-operation

See open biopsy under local anaesthesia.

Protocol 7: MESA

Indication

Patients with OA with normal spermatogenesis who wish to have epididymal sperm cryopreserved. The main drawback of MESA is that it is an invasive and expensive procedure requiring a basic knowledge of epididymal anatomy and of micro-surgical techniques. However, the major benefit of this procedure is its diagnostic power: a full scrotal exploration can be performed and, whenever indicated, a vasoepididymostomy may be performed concomitantly. Furthermore, the number of spermatozoa retrieved is high, which facilitates cryopreservation.

Caveat

When MESA is performed in CBAVD patients, a preliminary screening for mutations of the cystic fibrosis (CF) gene is mandatory in both the male CBAVD patient and his partner, since mutations are found in 60%–70% of CBAVD patients without congenital renal malformations. If the female partner is found to be a carrier of a CF gene mutation, pre-implantation genetic diagnosis should be proposed. Even where only the man is a carrier of a CF mutation, the couple has to be informed of the risk of having a boy with a genital CF phenotype with CBAVD.

Patient preparation

As for PESA (see Protocol 3).

MESA procedure

MESA can be performed during any scrotal exploration taking place even long before the ICSI treatment is scheduled or in a satellite centre (e.g. by a surgeon not involved in assisted reproduction).

Using an operating microscope, the epididymis is carefully dissected and after haemostasis. Using bipolar coagulation, a distended epididymal tubule is longitudinally opened by microscissors through a small opening in the serosa. The proximal corporal or distal head region of the epididymis is opened first. The epididymal fluid is aspirated by means of a disposable tip from an intravenous cannula mounted on a 1-mL syringe filled with 0.1 mL HEPES-buffered Earle's medium supplemented with 0.4%

human serum albumin. The aspirated epididymal fluid is then transferred into a Falcon test tube, which is filled with 0.9 mL of this Earle's medium. When motile spermatozoa are recovered, as assessed by peri-operative microscopic examination of the aspirates, no further epididymal incision is made and a maximum of fluid is aspirated. If microscopic assessment does not show any motile sperm cells, a more proximal incision is made until motile sperm cells are found. In some instances, centrifugation ($1800 \times g$, five minutes) of the epididymal aspirates is needed in order to observe spermatozoa under the microscope. In cases where no motile spermatozoa are recovered, a testicular biopsy is taken for sperm recovery. The sperm suspension is further prepared and kept in the incubator until the moment of intracytoplasmic injection or cryopreservation.

Patient care post-operation

Same as for TESE under general anaesthesia (see Protocol 6).

Requirements

- An assistant and a runner
- Needle holder Mayo-Hegar (20-642-16 Martin)
- Straight Mayo scissors (11-180-15 Martin)
- Monopolar pencil and cord (E 2502 Valleylab)
- Bipolar pincet and cord (4055 Valleylab)
- Tube holders (2x) (708130 Mölnlycke) to fix cords on drape (bipolar cord off head end, pencilcord off foot end)
- Micro-scissors (OP 5503 V-Mueller)
- Micro-needle holder (GU 8170 V-Meuller)
- Jeweller's forceps (3x) (E 1947 Storz)
- (72 BD 330 Aesculaep)
- Curved blunt scissors (11-939-14 Martin)
- 1 cc syringe (4x) (Air-tite K-ATS-1000 Cook) with or 22 ga medicut (8888 100 107 Argyle) or Cook aspiration CT (K Sal 400 300 Cook)
- Micro-Adson pincet with teeth (2x) (12-406-12 Martin)
- Knife handle with blades nr 15 (0505 Swann-Morton)
- Knife handle with blades nr 11 (0503 Swann-Morton)
- NaCl 0.9% 500 mL (B1323 Baxter) with 2500 IU Heparin Novo
- (Heparin Novo Nordisk Pharma)
- Syringes 20 cc (2x) (SS 20 ES Terumo) with 22 ga Medicut tip (8888 100107 Argyle)
- Swabs 10 x 10 (35813 Hartmann)
- Tip cleaner
- (Surgikos 4315 Johnson-Johnson)
- Micro-sponges (NDC 8065-1000-02 Alcon)
- Sutures
- Ethilon 9/0 (W 1769 Ethicon)
- Vicryl 3/0 (JV 497 Ethicon)
- Microscope
- Surgical operating and diagnostic microscope Wild M 691 with 180° positioning for doctor and assistant and optical eyepiece opposite each other
- (M 691 Leica)
- Achromatic lens f = 200 mm (M 382162 Leica)

Protocol 8: Testicular sperm cryopreservation

SF protocol

- $-1^{\circ}\text{C}/\text{minute}$ until a temperature of 5°C is reached
- $-10^{\circ}\text{C}/\text{minute}$ until a temperature of -80°C is reached
- Plunge the sample in liquid nitrogen (-196°C) [154]

Conventional SF protocols take approximately one hour to complete and can be automated for more precise temperature regulation using programmable controlled-rate freezers [148]. While these programmable freezers reduce the physical constraints on laboratory personnel, they are expensive and time-consuming to oversee.

RF protocol

- The spermatozoa are loaded directly into 0.25-mL straws and incubated at 4°C for 10 minutes
- The straws are rapidly frozen by positioning the straws 15–20 cm above the liquid nitrogen to expose the straws to -80°C for 15 minutes
- The straws are immersed in liquid nitrogen (-196°C)

URF protocol

- $-10^{\circ}\text{C}/\text{minute}$ by exposing cryo-straws to liquid nitrogen vapor 10 cm above the liquid nitrogen surface for 10 minutes
- Immersing the straws in liquid nitrogen (-196°C) [155]

REFERENCES

1. Palermo G, Joris K, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*. 1992;340:17–8.
2. Van Steirteghem AC, Nagy Z, Joris H, Liu J, Staessen C, Smits J, Wisanto A, Devroey P. Higher fertilization and implantation rates after intracytoplasmic sperm injection. *Hum Reprod*. 1993;8:1061–66.
3. Craft I, Benett V, Nicholson N. Fertilising ability of testicular spermatozoa (letter). *Lancet*. 1993;342:864.
4. Schoysman R, Vanderzwalmen P, Nijs M, Segal L, Segal-Bertin G, Geerts I, Roosendaal E, Schoysman D. Pregnancy after fertilisation with human testicular spermatozoa. *Lancet*. 1993;342:1237.
5. Devroey P, Liu J, Nagy Z, Goosens A, Tournaye H, Camus M, Van Steirteghem AC, Silber S. Pregnancies after testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermia. *Hum Reprod*. 1995;10:1457–60.
6. Tournaye H, Camus M, Goosens A, Nagy Z, Silber S, Van Steirteghem AC, Devroey P. Recent concepts in the management of infertility because of non-obstructive azoospermia. *Hum Reprod*. 1995;10(Suppl 1):115–9.
7. Jarow JP, Espeland MA, Lipschultz LI. Evaluation of the azoospermic patients. *J Urol*. 1989;142:62.
8. Matsumiya K, Namiki M, Takahara S, et al. Clinical study of azoospermia. *Int J Androl*. 1994;17:140–2.
9. Rastrelli G, Corona G, Mannucci E, Maggi M. Factors affecting spermatogenesis upon gonadotropin-replacement therapy: A meta-analytic study. *Andrology*. 2014;2:794–808.
10. Skakkebæk NE, Lindahl-Jacobsen R, Levine H, Andersson AM, Jørgensen N, Main KM, Lidegaard Ø, Priskorn L, Holmboe SA, Bräuner EV, Almstrup K, Franca LR, Znaor A, Kortenkamp A, Hart RJ, Juul A. Environmental factors in declining human fertility. *Nat Rev Endocrinol*. 2022;18(3):139–57.
11. Tournaye H, Camus M, Vandervorst M, Nagy Z, Joris H, Van Steirteghem A, Devroey P. Surgical sperm retrieval for intracytoplasmic sperm injection. *Int J Androl*. 1997;20(Suppl 3):69–73.
12. Tournaye H. Gamete source and manipulation. *Current Practices and Controversies in Assisted Reproduction—Report of a WHO Meeting*. Vayena E, Rowe P J, Griffin P D (eds.). World Health Organization Geneva. 2002; pp. 83–101.
13. Van Steirteghem A, Tournaye H, Joris H, Filicori M, Flamigni C, Van Landuyt L, Verheyen G. In *Treatment of Infertility: The New Frontiers. Proceedings of the Conference*, January 22–24, 1998, Filicori M, Flamigni C (eds.). Communications Media for Education Inc., Boca Raton, Florida.

14. Ron-El R, Strassburger D, Friedler S, et al. Extended sperm preparation: An alternative to testicular sperm extraction in non-obstructive azoospermia. *Hum Reprod.* 1997;12:1222–6.
15. Swanton A, Itani A, McVeigh E, Child T. Azoospermia: Is a simple centrifugation indicated? A national survey of practice and the Oxford experience. *Fertil Steril.* 2007;88:374–8.
16. Kafetsoulis A, Brackett N, Ibrahim E, Attia G, Lynne C. Current trends in the treatment of infertility in men with spinal cord injury. *Fertil Steril.* 2006;86:781–9.
17. O'Kelly F, Manecksha RP, Cullen IM, McDermott TE, Flynn R, Grainger R. Electroejaculatory stimulation and its implications for male infertility in spinal cord injury: A short history through four decades of sperm retrieval (1975–2010). *J Urology.* 2011;77:1349–52.
18. Hirsh A, Mills C, Tan SL, et al. Pregnancy using spermatozoa aspirated from the vas deferens in a patient with ejaculatory failure due to spinal injury. *Hum Reprod.* 1993;8:89–90.
19. Fan LW, Shao IH, Hsieh ML. The simple solution for infertile patients with aspermia in the modern era of assisted reproductive technique. *Urol Sci.* 2020;31:277–81.
20. Mehta A, Sigman M. Management of the dry ejaculate: A systematic review of aspermia and retrograde ejaculation. *Fertil Steril.* 2015;104:1074–81.
21. Soeterik TF, Veenboer PW, Lock TM. Electroejaculation in psychogenic anejaculation. *Fertil Steril.* 2014;101:1604–8.
22. Tur-Kaspa I, Segal S, Moffa F, Massobrio M, Meltzer S. Viagra for temporary erectile dysfunction during treatments with assisted reproductive technologies. *Hum Reprod.* 1999;14:1783–4.
23. Engin-Ustun Y, Korkmaz C, Duru NK, Baser I. Comparison of three sperm retrieval techniques in spinal cord-injured men: Pregnancy outcome. *Gynecol Endocrinol.* 2006;22:252–5.
24. Kamischke A, Nieschlag E. Update on medical treatment of ejaculatory disorders. *Int J Androl.* 2002;25:333–44.
25. Chéhensse C, Bahrami S, Denys P, Clément P, Bernabé J, Giuliano F. The spinal control of ejaculation revisited: A systematic review and meta-analysis of anejaculation in spinal cord injured patients. *Hum Reprod Update.* 2013;19:507–26.
26. Sonksen J, Fode M, Lochner-Ernst D, Ohl DA. Vibratory ejaculation in 140 spinal cord injured men and home insemination of their partners. *Spinal Cord.* 2012;50:63–6.
27. Meng X, Fan L, Liu J, Wang T, Yang J, Wang J, Wang S, Ye Z. Fresh semen quality in ejaculates produced by nocturnal emission in men with idiopathic anejaculation. *Fertil Steril.* 2013;100:1248–52.
28. Halstead LS, Vervoort S, Seager S. Rectal probe electrostimulation in the treatment of anejaculatory spinal cord injured men. *Paraplegia.* 1987;25:120–9.
29. McGuire C, Manecksha RP, Sheils P, McDermott TED, Grainger R, Flynn R. Electroejaculatory stimulation for male infertility secondary to spinal cord injury: The Irish experience in national rehabilitation Hospital. *J Urology.* 2011;77(1):83–7.
30. Kathiresan AS, Ibrahim E, Aballa TC, Attia GR, Ory SJ, Hoffman DI, Maxson WS, Barrionuevo MJ, Lynne CM, Brackett NL. Comparison of *in vitro* fertilization/intracytoplasmic sperm injection outcomes in male factor infertility patients with and without spinal cord injuries. *Fertil Steril.* 2011;96(3):562–6.
31. Tournaye H. Surgical sperm recovery for intracytoplasmic sperm injection: Which method is to be preferred? *Fertil Steril.* 2011;96(3):562–6.
32. Shah R. Surgical sperm retrieval: Techniques and their indications. *Indian J Urol.* 2011;27:102–9.
33. Uwin V, De Brucker S, De Brucker M, Vloeberghs V, Drakopoulos P, Santos-Ribeiro S, Tournaye H. Erratum. Pregnancy after vasectomy: Surgical reversal or assisted reproduction? *Hum Reprod.* 2018;33:1218–27.
34. Gangrade B. Cryopreservation of testicular and epididymal sperm: Techniques and clinical outcomes of assisted conception. *Clinics.* 2013;68:131–40.
35. Tournaye H, Merdad T, Silber S. No differences in outcome after intracytoplasmic sperm injection with fresh or with frozen-thawed epididymal sperm. *Hum Reprod.* 1999;14:101–6.
36. Nicopoullos J, Gilling-Smith C, Almeida P, et al. Use of surgical sperm retrieval in azoospermic men: A meta-analysis. *Fertil Steril.* 2004;82:691–701.
37. Ramos L, Kleingeld P, Meuleman E, van Kooy R, Kremer J, Braat D, Wetzel A. Assessment of DNA fragmentation of spermatozoa that were surgically retrieved from men with obstructive azoospermia. *Fertil Steril.* 2002;77:233–7.
38. Yafi F, Zini A. Percutaneous epididymal sperm aspiration for men with obstructive azoospermia: Predictors of successful sperm retrieval. *Urology.* 2013;82:341–4.
39. Gorgy A, Meniru GI, Bates S, Craft IL. Percutaneous epididymal sperm aspiration and testicular sperm aspiration for intracytoplasmic sperm injection under local anesthesia. *Assist Reprod Rev.* 1998;8:79–93.
40. van Wely M, Barbey N, Meissner A, Repping S, Silber SJ. Live birth rates after MESA or TESE in men with obstructive azoospermia: Is there a difference? *Hum Reprod.* 2015;30:761–6.
41. Tournaye H, Clasen K, Aytoz A, et al. Fine needle aspiration versus open biopsy for testicular sperm recovery: A controlled study in azoospermic patients with normal spermatogenesis. *Hum Reprod.* 1998;13:901–4.
42. Morey AF, Deshon GE Jr, Rozanski TA, Dresner ML. Technique of biopsy gun testis needle biopsy. *Urology.* 1993;42:325–6.
43. Donoso P, Tournaye H, Devroey P. Which is the best sperm retrieval technique for non-obstructive azoospermia? A systematic review. *Hum Reprod Update.* 2007;13:539–49.
44. Corona G, Minhas S, Giwercman A, Bettocchi C, Dinkelmann-Smit M, Dohle G, Fusco F, Kadioglu A, Kliensch S, Kopa Z, Krausz C, Pelliccione F, Pizzocaro A, Rassweiler J, Verze P, Vignozzi L, Weidner W, Maggi M, Sofikitis N. Sperm recovery and ICSI outcomes in men with non-obstructive azoospermia: A systematic review and meta-analysis. *Hum Reprod Update.* 2019;25:733–57.
45. Tournaye H, Liu J, Nagy Z, et al. Correlation between testicular histology and outcome after intracytoplasmic sperm injection using testicular sperm. *Hum Reprod.* 1996;11:127–32.
46. Ezech UI, Taub NA, Moore HD, Cooke ID. Establishment of predictive variables associated with testicular sperm retrieval in men with nonobstructive azoospermia. *Hum Reprod.* 1999;14:1005–12.
47. Bonarriba CR, Burgués JP, Vidaña V, Ruiz X, Pizá P. Predictive factors of successful sperm retrieval in azoospermia. *Actas Urol Esp.* 2013;37:266–72.
48. Tournaye H, Verheyen G, Nagy P, et al. Are there any predictive factors for successful testicular sperm recovery? *Hum Reprod.* 1997;12:80–6.
49. Mitchell V, Robin G, Boitrelle F, Massart P, Marchetti C, Marcelli F, Rigot JM. Correlation between testicular sperm extraction outcomes and clinical, endocrine and testicular histology parameters in 120 azoospermic men with normal serum FSH levels. *Int J Androl.* 2011;34:299–305.
50. Ballesca JL, Balasch J, Calafell JM, et al. Serum inhibin b determination is predictive of successful testicular sperm extraction in men with non-obstructive azoospermia. *Hum Reprod.* 2000;15:1734–8.
51. Vernaeve V, Tournaye H, Schietecatte J, et al. Serum inhibin b cannot predict testicular sperm retrieval in patients with non-obstructive azoospermia. *Hum Reprod.* 2002;17:971–6.
52. Tunc L, Kirac M, Gurocak S, Yucel A, Kupeli B, Alkibay T, Bozkirli I. Can serum inhibin b and FSH levels, testicular histology and volume predict the outcome of testicular sperm extraction in patients with non-obstructive azoospermia? *Int Urol Nephrol.* 2006;38:629–35.
53. Huang X, Bai Q, Yan LY, Zhang QF, Geng L, Qiao J. Combination of serum inhibin b and follicle-stimulating hormone levels cannot improve the diagnostic accuracy on testicular sperm extraction outcomes in Chinese non-obstructive azoospermic men. *Chin Med J (Engl).* 2012;125:2885–9.

54. Ma Y, Chen B, Wang H, Hu K, Huang Y. Prediction of sperm retrieval in men with non-obstructive azoospermia using artificial neural networks: Leptin is a good assistant diagnostic marker. *Hum Reprod.* 2011;26:294–8.
55. Boitrelle F, Robin G, Marcelli F, Albert M, Leroy-Martin B, Dewailly D, Rigot J, Mitchell V. A predictive score for testicular sperm extraction quality and surgical intra-cytoplasmic sperm injection outcome in non-obstructive azoospermia: A retrospective study. *Hum Reprod.* 2011;26:3215–21.
56. Tournaye H. How to predict fatherhood for men with non-obstructive azoospermia opting for TESE-ICSI? *Hum Reprod.* 2011;26:3213–4.
57. Zeadna A, Khateeb N, Rokach L et al. Prediction of sperm extraction in non-obstructive azoospermia patients: A machine learning perspective. *Hum Reprod.* 2020;35(7):1505–14.
58. Har-Toov J, Eytan O, Hauser R, Yavetz H, Elad D, Jaffa AJ. A new power doppler ultrasound guiding technique for improved testicular sperm extraction. *Fertil Steril.* 2004;81:430–4.
59. Herwig R, Tosun K, Pinggera GM, Soelder E, Moeller KT, Pallwein L, Frauscher E, Bartsch G, Wildt L, Illmensee K. Tissue perfusion essential for spermatogenesis and outcome of testicular sperm extraction (TESE) for assisted reproduction. *J Assist Reprod Genet.* 2004;21:175–80.
60. Ntorkou A, Tsili A, Goussia A et al. Testicular apparent diffusion coefficient and magnetization transfer ratio: Can this MRI be used to predict successful sperm retrieval in nonobstructive azoospermia? *AJR Am J Roentgenol.* 2019;213(3):610–8.
61. Bettella A, Ferlin A, Menegazzo M, Ferigo M, Tavolini M, Bassi PF, Foresta C. Testicular fine needle aspiration as a diagnostic tool in non-obstructive azoospermia. *Asian J Androl.* 2005;7:289–94.
62. Beliveau ME, Turek PJ. The value of testicular ‘mapping’ in men with non-obstructive azoospermia. *Asian J Androl.* 2011;13: 225–30.
63. Brandell RA, Mielenik A, Liotta D, Ye Z, Veeck LL, Palermo GD, Schlegel PN. AZFb deletions predict the absence of spermatozoa with testicular sperm extraction: Preliminary report of a prognostic genetic test. *Hum Reprod.* 1998;13:2812–5.
64. Stouffs K, Vloeberghs V, Gheldof A, Tournaye H, Seneca S. Are AZFb deletions always incompatible with sperm production? *Andrology.* 2017;5:691–4.
65. Terribile M, Stizzo M, Manfredi C, Quattrone C, Bottone F, Giordano DR, Bellastella G, Arcaniolo D, De Sio M. 46,XX testicular disorder of sex development (DSD): A case report and systematic review. *Medicina (Kaunas).* 2019;55:371.
66. Silber S, Nagy Z, Devroey P, Tournaye H, Van Steirteghem AC. Distribution of spermatogenesis in the testicles of azoospermic men: The presence or absence of spermatids in the testes of men with germinal failure. *Hum Reprod.* 1997;12:2422–8.
67. Gil-Salom M, Minguez Y, Rubio C, de los Santos MJ, Remohi J, Pellicer A. Efficacy of intracytoplasmic sperm injection using testicular spermatozoa. *Hum Reprod.* 1995;10:3166–70.
68. Hauser R, Botchan A, Amit A, Ben Yosef D, Gamzu R, Paz G, Lessing JB, Yogeve L, Yavetz H. Multiple testicular sampling in non-obstructive azoospermia—Is it necessary? *Hum Reprod.* 1998;13:3081–5.
69. Witt MA, Richard JR, Smith SE, Rhee EH, Tucker MJ. The benefit of additional biopsy sites when performing testicular sperm extraction in nonobstructive azoospermia. *Fertil Steril.* 1997;67: S79–80.
70. Verhaeghe P, Verheyen G, Goossens A, et al. How successful is repeat testicular sperm extraction in patients with azoospermia? *Hum Reprod.* 2006;21:1551–4.
71. Ezeb UIO, Moore HDM, Cooke ID. Correlation of testicular sperm extraction with morphological, biophysical and endocrine profiles in men with azoospermia due to primary gonadal failure. *Hum Reprod.* 1998;13:3066–74.
72. Schlegel P, Su LM. Physiological consequences of testicular sperm extraction. *Hum Reprod.* 1997;12:1688–92.
73. Ron-El R, Strauss S, Friedler S, Strassburger D, Komarovsky D, Raziel A. Serial sonography and colour flow doppler imaging following testicular and epididymal sperm extraction. *Hum Reprod.* 1998;13:3390–3.
74. Schlegel PN, Li PS. Microdissection TESE: Sperm retrieval in non-obstructive azoospermia. *Hum Reprod Update.* 1998;4:439.
75. Ishikawa T, Nose R, Yamaguchi K, et al. Learning curves of microdissection testicular sperm extraction for nonobstructive azoospermia. *Fertil Steril.* 2010;94:1008–11.
76. Kalsi JS, Shah P, Thum Y, Munneer A, Ralph DJ, Minhas S. Salvage micro-dissection testicular sperm extraction; Outcome in men with non-obstructive azoospermia with previous failed sperm retrievals. *BJU Int.* 2014;116:460–5.
77. Bernie A, Mata D, Ramasamy R, Schlegel P. Comparison of microdissection sperm extraction, conventional testicular sperm extraction, and testicular sperm aspiration for nonobstructive azoospermia. *Fertil Steril.* 2015;104:1099–103.
78. Deruyver Y, Vanderschueren D, Van der Aa F. Outcome of microdissection TESE compared with conventional TESE in non-obstructive azoospermia: A systematic review. *Andrology.* 2014;2:20–4.
79. Berookhim BM, Palermo GD, Zaninovic N, Rosenwaks Z, Schlegel PN. Microdissection testicular sperm extraction in men with Sertoli cell-only testicular histology. *Fertil Steril.* 2014;102:1282–6.
80. Vloeberghs V, Verheyen G, Haentjens P, Goossens A, Polyzos NP, Tournaye H. How successful is TESE-ICSI in couples with non-obstructive azoospermia? *Hum Reprod.* 2015;30:1790–6.
81. Corona G, Minhas S, Giwercman A, Bettocchi C, Dinkelmann-Smit M, Dohle G, Fusco F, Kadioglou A, Kliesch S, Kopa Z, et al. Sperm recovery and ICSI outcomes in men with non-obstructive azoospermia: A systematic review and meta-analysis. *Hum Reprod Update.* 2019;25:733–757.
82. Esteves SC, Ramasamy R, Colpi GM, Carvalho JF, Schlegel PN. Sperm retrieval rates by micro-TESE versus conventional TESE in men with non-obstructive azoospermia—the assumption of independence in effect sizes might lead to misleading conclusions. *Hum Reprod Update.* 2020;26:603–5.
83. Corona G, Minhas S, Bettocchi C, Krausz C, Pizzocaro A, Vena W, Maggi M, Sofikitis N. Reply: Sperm retrieval rates by micro-TESE versus conventional TESE in men with non-obstructive azoospermia—the assumption of independence in effects sizes might lead to misleading conclusions. *Hum Reprod Update.* 2020;26(4): 606–9.
84. Verheyen G, Verhaeghe P, Van Landuyt L, et al. Should diagnostic testicular sperm retrieval followed by cryopreservation for later ICSI be the procedure of choice for all patients with non-obstructive azoospermia? *Hum Reprod.* 2004;19:2822–30.
85. Ohlander S, Hotaling J, Kirshenbaum E, et al. Impact of fresh versus cryopreserved testicular sperm upon intracytoplasmic sperm injection pregnancy outcomes in men with azoospermia due to spermatogenic dysfunction: A meta-analysis. *Fertil Steril.* 2014;101:344–9.
86. Tournaye H, Staessen C, Liebaers I, et al. Testicular sperm recovery in 47, XXY klinefelter patients. *Hum Reprod.* 1996;11: 1644–9.
87. Dávila Garza SA, Patrizio P. Reproductive outcomes in patients with male infertility because of Klinefelter’s syndrome, Kartagener’s syndrome, round-head sperm, dysplasia fibrous sheath, and ‘stump’ tail sperm: An updated literature review. *Curr Opin Obstet Gynecol.* 2013;25:229–46.
88. Tournaye H, Camus M, Vandervorst M et al. Sperm retrieval for ICSI. *Int J Androl.* 1997;20(Suppl 3):69–73.
89. Corona G, Pizzocaro A, Lanfranco F, Garolla A, Pelliccione F, Vignozzi L, Ferlin A, Foresta C, Jannini EA, Maggi M, Lenzi A, Pasquali D, Francavilla S, Klinefelter ItaliaN Group (KING). Sperm recovery and ICSI outcomes in klinefelter syndrome: A systematic review and meta-analysis. *Hum Reprod Update.* 2017;23(3):265–275.

90. Okada H, Goda K, Yamamoto Y, Sofikitis N, Miyagawa I, Mio Y, Koshida M, Horie S. Age as a limiting factor for successful sperm retrieval in patients with nonmosaic Klinefelter's syndrome. *Fertil Steril.* 2005;84:1662–4.
91. Staessen C, Coonen E, Van Assche E, et al. Preimplantation diagnosis for x and y normality in embryos from three klinefelter patients. *Hum Reprod.* 1996;11:1650–3.
92. Staessen C, Tournaye H, Van Assche E, et al. Preimplantation diagnosis in 47, XXY klinefelter patients. *Hum Reprod Update.* 2003;9:319–30.
93. Scirano RB, Luna Hisano CV, Rahn MI, et al. Focal spermatogenesis originates in euploid germ cells in classical klinefelter patients. *Hum Reprod.* 2009;24:2353–60.
94. Vloeberghs V, Verheyen G, Santos-Ribeiro S, Staessen C, Verpoest W, Gies I, Tournaye H. Is genetic fatherhood within reach for all azoospermic klinefelter men? *PLoS One.* 2018 Jul 25; 13(7):e0200300.
95. Tournaye H, Dohle GR, Barratt CL. Fertility preservation in men with cancer. *Lancet.* 2014;384:1295–301.
96. Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, Mitchell RT, Pennings G, Rives N, Tournaye H, van Pelt AM, Eichenlaub-Ritter U, Schlatt SESRE Task Force On Fertility Preservation In Severe Diseases. A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. *Hum Reprod.* 2015;30:2463–75.
97. Rosenlund B, Sjöblom P, Tornblom M, et al. *In-vitro* fertilization and intracytoplasmic sperm injection in the treatment of infertility after testicular cancer. *Hum Reprod.* 1998;13:414–8.
98. Baniel J, Sella A. Sperm extraction at orchietomy for testis cancer. *Fertil Steril.* 2001;75:260–2.
99. Schrader M, Muller M, Straub B, et al. Testicular sperm extraction in azoospermic patients with gonadal germ cell tumors prior to chemotherapy—A new therapy option. *Urology.* 2003;61:421–5.
100. Damani MN, Masters V, Meng MV, et al. Postchemotherapy ejaculatory azoospermia: Fatherhood with sperm from testis tissue with intracytoplasmic sperm injection. *J Clin Oncol.* 2002;15:930–6.
101. Hsiao W, Stahl PJ, Osterberg EC, Nejat E, Palermo GD, Rosenwaks Z, Schlegel PN. Successful treatment of postchemotherapy azoospermia with microsurgical testicular sperm extraction: The Weill Cornell experience. *J Clin Oncol.* 2011;29:1607–11.
102. Hussein A, Ozgok Y, Ross L, Rao P, Niederberger C. Optimization of spermatogenesis-regulating hormones in patients with non-obstructive azoospermia and its impact on sperm retrieval: A multicentre study. *BJU Int.* 2013;111:E110–4.
103. Schlegel P. Aromatase inhibitors for male infertility. *Fertil Steril.* 2012;98:1359–62.
104. Reifsnyder J, Ramasamy R, Hussein J, Schlegel P. Role of optimizing testosterone before microdissection testicular sperm extraction in men with nonobstructive azoospermia. *J Urol.* 2012;188:532–7.
105. Mehta A, Bolyakov A, Roosma J, Schlegel P, Paduch D. Successful testicular sperm retrieval in adolescents with klinefelter syndrome treated with at least 1 year of topical testosterone and aromatase inhibitor. *Fertil Steril.* 2013;100:970–4.
106. Kyrou D, Kosmas I, Popovic-Todorovic B, Donoso P, Devroey P, Fatemi H. Ejaculatory sperm production in non-obstructive azoospermic patients with a history of negative testicular biopsy after the administration of an aromatase inhibitor: Report of two cases. *Eur J Obstet Gynecol Reprod Biol.* 2014;173:120–1.
107. Alrabeeah K, Addar A, Alothman A, Melha SA, Alkhayal A. Effect of hormonal manipulation using clomiphene citrate prior to microdissection testicular sperm retrieval. *Urol Ann.* 2021;13:254–7.
108. Friedler S, Raziel A, Schachter M, Strassburger D, Bern O, Ron-El R. Outcome of first and repeated testicular sperm extraction in patients with non-obstructive azoospermia. *Hum Reprod.* 2002;17:2356–61.
109. Kamal A, Fahmy I, Mansour R, Abou-Setta A, Serour G, Aboulghar M. Outcome of repeated testicular sperm extraction and ICSI in patients with non-obstructive azoospermia. *MEFSJ.* 2004;9:42–6.
110. Hauser R, Yoge L, Paz G, Yavetz H, Azem F, Lessing JB, Botchan A. Comparison of efficacy of two techniques for testicular sperm retrieval in nonobstructive azoospermia: Multifocal testicular sperm extraction versus multifocal testicular sperm aspiration. *J Androl.* 2006;27:28–33.
111. Rosenlund B, Kvist U, Ploen L, Lundh Rozell B, Sjöblom P, Hillensjö T. A comparison between open and percutaneous needle biopsies in men with azoospermia. *Hum Reprod.* 1998;13:1266–71.
112. Friedler S, Raziel A, Strassburger D, et al. Testicular sperm retrieval by percutaneous fine needle sperm aspiration compared with testicular sperm extraction by open biopsy in men with non-obstructive azoospermia. *Hum Reprod.* 1997;12:1488–93.
113. Ezeh UIO, Moore HDM, Cooke ID. A prospective study of multiple needle biopsies versus a single open biopsy for testicular sperm extraction in men with non-obstructive azoospermia. *Hum Reprod.* 1998;13:3075–80.
114. Novero V, Goossens A, Tournaye H, Silber S, Van Steirteghem A, Devroey P. Seminoma discovered in two males undergoing successful testicular sperm extraction for intracytoplasmic sperm injection. *Fertil Steril.* 1996;65:1015–54.
115. Ramasamy R, Reifsnyder JE, Bryson C, Zaninovic N, Liotta D, Cook CA, Hariprasad J, Weiss D, Neri Q, Palermo GD, et al. Role of tissue digestion and extensive sperm search after microdissection testicular sperm extraction. *Fertil Steril.* 2011;96:299–302.
116. Nagy P, Verheyen G, Tournaye H, Devroey P, Van Steirteghem A. An improved treatment procedure for testicular biopsy offers more efficient sperm recovery: Case series. *Fertil Steril.* 1997;68:376–9.
117. Popal W, Nagy ZP. Laboratory processing and intracytoplasmic sperm injection using epididymal and testicular spermatozoa: What can be done to improve outcomes? *Clinics (Sao Paulo).* 2013;68(Suppl 1):125–30.
118. Crabbé E, Verheyen G, Tournaye H, Van Steirteghem A. The use of enzymatic procedures to recover testicular sperm. *Hum Reprod.* 1997;12:1682–7.
119. Baukloh V. Retrospective multicentre study on mechanical and enzymatic preparation of fresh and cryopreserved testicular biopsies. *Hum Reprod.* 2002;17:1788–1794.
120. Aydos K, Demirel LC, Baltaci V, Unlü C. Enzymatic digestion plus mechanical searching improves testicular sperm retrieval in nonobstructive azoospermia cases. *Eur J Obstet Gynecol Reprod Biol.* 2005;120:80–6.
121. Angelopoulos T, Adler A, Krey L, Licciardi F, Noyes N, McCullough A. Enhancement or initiation of testicular sperm motility by *in vitro* culture of testicular tissue. *Fertil Steril.* 1999;71:240–3.
122. Tasdemir I, Tasdemir M, Tavukcuoglu S. Effect of pentoxifylline on immotile testicular spermatozoa. *J Assisted Reprod Genet.* 1998;15:90–2.
123. Terriou P, Hans E, Giorgetti C, Spach JL, Salzmann J, Urrutia V, Roulier R. Pentoxifylline initiates motility in spontaneously immotile epididymal and testicular spermatozoa and allows normal fertilization, pregnancy, and birth after intracytoplasmic sperm injection. *J Assist Reprod Genet.* 2000;17:194–9.
124. Griveau JF, Lobel B, Laurent MC, Michardière L, Le Lannou D. Interest of pentoxifylline in ICSI with frozen-thawed testicular spermatozoa from patients with non-obstructive azoospermia. *Reprod Biomed Online.* 2006;12(1):14–8.
125. Schlegel PN, Su LM. Physiological consequences of testicular sperm extraction. *Hum Reprod.* 1997;12(8):1688–92.
126. Verheyen G, De Croo I, Tournaye H, Pletincx I, Devroey P, van Steirteghem AC. Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. *Hum Reprod.* 1995;10(11):2956–9.
127. Oates RD, Mulhall J, Burgess C, Cunningham D, Carson R. Fertilization and pregnancy using intentionally cryopreserved testicular tissue as the sperm source for intracytoplasmic sperm injection in 10 men with non-obstructive azoospermia. *Hum Reprod.* 1997;12(4):734–9.

128. Ostad M, Liotta D, Ye Z, Schlegel PN. Testicular sperm extraction for nonobstructive azoospermia: Results of a multibiopsy approach with optimized tissue dispersion. *Urology*. 1998;52(4):692–6.
129. Popal W, Nagy ZP. Laboratory processing and intracytoplasmic sperm injection using epididymal and testicular spermatozoa: What can be done to improve outcomes? *Clinics (Sao Paulo)*. 2013;68(Suppl 1):125–30.
130. Wober M, Ebner T, Steiner SL et al. A new method to process testicular sperm: Combining enzymatic digestion, accumulation of spermatozoa, and stimulation of motility. *Arch Gynecol Obstet*. 2015;291(3):689–94.
131. Ramasamy R, Reifsnyder JE, Bryson C et al. Role of tissue digestion and extensive sperm search after microdissection testicular sperm extraction. *Fertil Steril*. 2011;96(2):299–302.
132. Aydos K, Demirel LC, Baltaci V, Unlu C. Enzymatic digestion plus mechanical searching improves testicular sperm retrieval in non-obstructive azoospermia cases. *Eur J Obstet Gynecol Reprod Biol*. 2005;120(1):80–6.
133. Crabbé E, Verheyen G, Tournaye H, Van Steirteghem A. The use of enzymatic procedures to recover testicular germ cells. *Hum Reprod*. 1997;12(8):1682–7.
134. World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. Geneva, Switzerland: World Health Organisation Press, 2021.
135. Baukloh V. Retrospective multicentre study on mechanical and enzymatic preparation of fresh and cryopreserved testicular biopsies. *Hum Reprod*. 2002;17(7):1788–94.
136. Pegg DE. The history and principles of cryopreservation. *Semin Reprod Med*. 2002;20(1):5–13.
137. Liu S, Li F. Cryopreservation of single-sperm: Where are we today? *Reprod Biol Endocrinol*. 2020;18(1):41.
138. Sawada Y, Ackerman D, Behrman SJ. Motility and respiration of human spermatozoa after cooling to various low temperatures. *Fertil Steril*. 1967;18(6):775–81.
139. Leibo SP. Fundamental cryobiology of mouse ova and embryos. *Ciba Found Symp*. 1977; (52): 69–96.
140. Gage AA, Baust JM, Baust JG. Experimental cryosurgery investigations *in vivo*. *Cryobiology*. 2009;59(3):229–43.
141. Anger JT, Gilbert BR, Goldstein M. Cryopreservation of sperm: Indications, methods and results. *J Urol*. 2003;170(4 Pt 1):1079–84.
142. Craft I, Tsirigotis M. Simplified recovery, preparation and cryopreservation of testicular spermatozoa. *Hum Reprod*. 1995;10(7): 1623–6.
143. Romero J, Remohi J, Minguez Y, Rubio C, Pellicer A, Gil-Salom M. Fertilization after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. *Fertil Steril*. 1996;65(4):877–9.
144. Ohlander S, Hotaling J, Kirshenbaum E, Niederberger C, Eisenberg ML. Impact of fresh versus cryopreserved testicular sperm upon intracytoplasmic sperm injection pregnancy outcomes in men with azoospermia due to spermatogenic dysfunction: A meta-analysis. *Fertil Steril*. 2014;101(2):344–9.
145. Rothman S. What is sperm banking? When and how is it (or should it be) used in humans? *Animals?* In: *Handbook of Andrology*, 2nd edition. Robaire B, Chan P (eds). Lawrence, KS: Allen Press, Inc., 2010, pp. 41–4.
146. Jeyendran RS, Gunawardana VK, Barisic D, Wentz AC. TEST-yolk media and sperm quality. *Hum Reprod Update*. 1995;1(1):73–9.
147. Nallella KP, Sharma RK, Allamaneni SS, Aziz N, Agarwal A. Cryopreservation of human spermatozoa: Comparison of two cryopreservation methods and three cryoprotectants. *Fertil Steril*. 2004;82(4):913–8.
148. Vutyavanich T, Piromlertamorn W, Nunta S. Rapid freezing versus slow programmable freezing of human spermatozoa. *Fertil Steril*. 2010;93(6):1921–8.
149. Tongdee P, Sukprasert M, Satirapod C, Wongkularb A, Choktanasiri W. Comparison of cryopreserved human sperm between ultra rapid freezing and slow programmable freezing: Effect on motility, morphology and DNA integrity. *J Med Assoc Thai*. 2015;98(Suppl 4):S33–42.
150. Trummer H, Tucker K, Young C, Kaula N, Meacham RB. Effect of storage temperature on sperm cryopreservation. *Fertil Steril*. 1998;70(6):1162–4.
151. Martinez-Soto JC, Garcia-Vazquez FA, Gumbao D, Landeras J, Gadea J. Assessment of two thawing processes of cryopreserved human sperm in pellets. *Cryobiology*. 2011;63(3):131–6.
152. AbdelHafez F, Bedaiwy M, El-Nashar SA, Sabanegh E, Desai N. Techniques for cryopreservation of individual or small numbers of human spermatozoa: A systematic review. *Hum Reprod Update*. 2009;15(2):153–64.
153. Huang C, Gan RX, Hu JL, Liu F, Hong Y, Zhu WB, Li Z. Clinical benefit for cryopreservation of single human spermatozoa for ICSI: A systematic review and meta-analysis. *Andrology*. 2022;10(1): 82–91.
154. Di Santo M, Tarozzi N, Nadalini M, Borini A. Human sperm cryopreservation: Update on techniques, effect on DNA integrity, and implications for ART. *Adv Urol*. 2012;2012:854837.
155. Stanic P, Tandara M, Sonicki Z, Simunic V, Radakovic B, Suchanek E. Comparison of protective media and freezing techniques for cryopreservation of human semen. *Eur J Obstet Gynecol Reprod Biol*. 2000;91(1):65–70.
156. Nagy Z, Liu J, Janssenwillen C, et al. Comparison of fertilization, embryo development and pregnancy rates after intracytoplasmic sperm injection using ejaculated, fresh and frozen-thawed epididymal and testicular spermatozoa. *Fertil Steril*. 1995;63:808–5.
157. Vernaev V, Tournaye H, Osmanagaoglu K, et al. Intracytoplasmic sperm injection with testicular spermatozoa is less successful in men with nonobstructive azoospermia than in men with obstructive azoospermia. *Fertil Steril*. 2003;79:529–33.
158. Esteves SC, Agarwal A. Reproductive outcomes, including neonatal data, following sperm injection in men with obstructive and nonobstructive azoospermia: Case series and systematic review. *Clinics (Sao Paulo)*. 2013;68(Suppl 1):141–50.
159. Levron J, Aviram-Goldring A, Madgar I, et al. Sperm chromosome abnormalities in men with severe male factor infertility who are undergoing *in-vitro* fertilization with intracytoplasmic sperm injection. *Fertil Steril*. 2001;76:479–84.
160. Platteau P, Staessen C, Michiels A, et al. Comparison of the aneuploidy frequency in embryos derived from testicular sperm extraction in obstructive and nonobstructive azoospermic men. *Hum Reprod*. 2004;19:1570–4.
161. Donoso P, Platteau P, Papanikolaou EG, et al. Does PGD for aneuploidy screening change the selection of embryos derived from testicular sperm extraction in obstructive and non-obstructive azoospermic men? *Hum Reprod*. 2006;21:2390–5.
162. Chen M, Wei S, Hu J, Quan S. Can comprehensive chromosome screening technology improve IVF/ICSI outcomes? A meta-analysis. *PLoS One*. 2015;10:e0140779.
163. Mazzilli R, Cimadomo D, Vaiarelli A, Capalbo A, Dovere L, Alviggi E, Dusi L, Foresta C, Lombardo F, Lenzi A, Tournaye H, Alviggi C, Rienzi L, Ubaldi FM. Effect of the male factor on the clinical outcome of intracytoplasmic sperm injection combined with preimplantation aneuploidy testing: Observational longitudinal cohort study of 1,219 consecutive cycles. *Fertil Steril*. 2017;108:961–972.
164. Schlegel PN. Nonobstructive azoospermia: A revolutionary surgical approach and results. *Semin Reprod Med*. 2009;27:165–70.
165. Osmanagaoglu K, Vernaev V, Kolibianakis E, Tournaye H, Camus M, Van Steirteghem A, Devroey P. Cumulative delivery rates after ICSI treatment cycles with freshly retrieved testicular sperm: A 7-year follow-up study. *Hum Reprod*. 2003;18:1836–40.
166. Giorgetti C, Chinchole JM, Hans E, Charles O, Franquebalme JP, Glowaczower E, Salzmann J, Terriou P, Roulier R. Crude cumulative delivery rate following ICSI using intentionally frozen-thawed testicular spermatozoa in 51 men with non-obstructive azoospermia. *Reprod Biomed Online*. 2005;11:319–24.
167. Dafopoulos K, Griesinger G, Schultze-Mosgau A, Orrief Y, Schöpper B, Nikolettos N, Diedrich K, Al-Hasani S. Cumulative pregnancy rate after ICSI with cryopreserved testicular tissue in non-obstructive azoospermia. *Reprod Biomed Online*. 2005;10:461–6.

168. Fedder J, Loft A, Parner ET, Rasmussen S, Pinborg A. Neonatal outcome and congenital malformations in children born after ICSI with testicular or epididymal sperm: A controlled national cohort study. *Hum Reprod.* 2013;28(1):230–40.
169. Woltringh GH, Besselink DE, Tillema AH, Hendriks JC, Kremer JA. Karyotyping, congenital anomalies and follow-up of children after intracytoplasmic sperm injection with non-ejaculated sperm: A systematic review. *Hum Reprod Update.* 2010;16:12–9.
170. Woltringh GH, Horvers M, Janssen AJ, Reuser JJ, de Groot SA, Steiner K, D'Hauwers KW, Wetzel AM, Kremer JA. Follow-up of children born after ICSI with epididymal spermatozoa. *Hum Reprod.* 2011;26(7):1759–67.
171. Vernaeve V, Bonduelle M, Tournaye H, et al. Pregnancy outcome and neonatal data on children born after ICSI with testicular sperm in obstructive and non-obstructive azoospermia. *Hum Reprod.* 2003;18:2093–7.
172. Belva F, De Schrijver F, Tournaye H, et al. Neonatal outcome of 724 children born after ICSI using non-ejaculated sperm. *Hum Reprod.* 2011;26:1752–8.

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EMBRYO TRANSFER TECHNIQUE

Leah Roberts and Jason Franasiak

Introduction

The procedure of embryo transfer is simple in description—insert the transfer catheter into the uterine cavity and deliver the embryo—however differences in technique can greatly affect rates of success. Of course, the embryo must be deposited in the correct location where the likelihood of implantation is highest without causing trauma to the receptive endometrium [1]. Studies have shown that the rates can differ between practitioners at a single clinic by as much as 37.3% [2]. Standardization of technique throughout a practice can remove this dependence on the physician performing the ET for success [3]. Transfer technique is a variable independent of the patient's intrinsic characteristics [2] that can be modified by implementing evidence-based [4] practice [3].

For trainees, this is a technique that has been proven to be teachable, with proficiency seen by the 15th transfer [5]. Practice has shifted with proof that trainees can be successfully trained to perform embryo transfers without any decrease in rates of success for the patients [6, 7].

Simulation training for embryo transfer has been developed in order to maximize the skill of trainees prior to performing their first live transfers and is now offered as a certificate course through the American Society for Reproductive Medicine [8, 9]. This allows for a highly realistic environment for less-experienced providers to hone their skills and learn from live feedback of their own transfer metrics without jeopardizing patients' outcomes [8]. This course has been validated in both easy and more difficult transfers and through all years of training [9].

Pre-transfer techniques

Many complementary therapies have been used during the embryo transfer cycle and just prior to transfer in order to improve outcomes. Acupuncture, massage, medical clowning, and transcutaneous electrical acupoint stimulation (TEAS) have all been studied in a randomized controlled fashion, with little associated risk and some benefits shown. Some of the limitations include the control groups and the overall low rates of implantation and live birth in some of the studies.

Endometrial scratch

Endometrial scratch describes a technique where intentional damage is done to the endometrium with the purpose of improving endometrial receptivity. A meta-analysis of endometrial scratch injury prior to first transfer was performed using data from seven studies ($n = 1354$) and showed no difference in outcomes [10]. A meta-analysis by the same group showed patients with one failed cycle (five studies) did not have any improvements in outcome with endometrial scratch, however those with two or more failed cycles (five studies) did have a higher live birth rate with a relative risk (RR) of 1.64 (CI 1.21–2.21). These studies had a large amount of heterogeneity, however, with a difference in number and time during the cycle of endometrial scratch and were

rated to be low quality [10, 11]. A Cochrane analysis of 38 clinical trials ($n = 8915$) also did not show any evidence to support the routine use of endometrial scratch [12].

Acupuncture

Acupuncture has been increasingly used as part of treatment, at various times during the cycle, with many small studies performed to evaluate its effectiveness with conflicting results. One four-armed well-powered randomized controlled trial (RCT) was performed to assess the benefits of laser acupuncture, needle acupuncture, sham laser acupuncture, and no treatment. It did show an improvement in implantation rates with laser acupuncture compared to the other three arms, however it was not powered to show a significant difference in live birth rate [13]. Additionally, a meta-analysis was performed looking at RCTs that evaluated the effects of acupuncture (manual, electrical, and laser) on IVF outcomes. There was significant heterogeneity between the studies, with differences in technique, time of commencement, control, location of acupuncture sites, and patient characteristics. Placebo was often not able to be assessed dependent on methods used although a few did perform sham acupuncture. Clinical pregnancy rates were increased with an odds ratio (OR) 1.22 ($p = 0.04$), however live birth rates were not shown to be increased [14]. This technique, however, offers little risk and can be reasonably offered as an adjunct to patients.

Transcutaneous electrical acupoint stimulation (TEAS)

There is one prospective randomized single-blinded trial of TEAS ($n = 309$) which showed increased live birth rate of 37.3% (one treatment on day of transfer) and 42.0% (two treatments, one day of and one 24 hours before) compared to 21.2% (mock treatment) ($p = 0.011$ and 0.002) [15].

Massage

Less work has been done in a rigorous fashion on massage, however, one retrospective observational study did show significantly higher birth rates (32% vs 20%; $p < 0.05$) in patients who underwent 30-minute, deep relaxation massage on an oscillating device compared to those who did not [16]. Suggested mechanisms of action include a reduction in stress, a reduction in uterine contractions, and possibly an enhancement of the blood flow in the abdominal region.

Oxytocin antagonist

Both decreased endometrial blood flow and increased uterine contractions have been linked to decreased implantation rates [17, 18]. As there are oxytocin receptors in the myometrium, endometrium, and blood vessels of the uterus around the time of implantation, there is a theoretical benefit to using an oxytocin antagonist in order to reduce the contractility and increase perfusion of the uterus [18]. Drugs that have been studied for this usage include IV atosiban, subcutaneous barusiban, and oral nolasiban [19].

One analysis has been performed looking at three RCTs performed by the same group with a total of 1836 patients who underwent frozen embryo transfer (FET) four hours after receiving the oxytocin antagonist nolasiban. Only the highest dose tested, 900 mg, was shown to have a 4.4% improvement in live birth rate compared to placebo, which was non-significant ($p = 0.053$) [18]. It is possible that certain subgroups may show a larger, statistically significant response to an oxytocin antagonist, so further research looking, for instance, at those with high contraction frequency may be warranted to best target this treatment to those who would most benefit from its implementation [17, 20].

Antibiotics

Several studies have shown a correlation between cervical colonization by pathogenic bacteria and decreased rates of live birth [21–23]. It would make sense then, that antibiotic treatment would reduce the pathogenic bacterial load, and therefore improve outcomes; however, this has not been proven by any studies. Several retrospective studies have shown no difference in live birth rates with or without doxycycline [24]. Only one RCT has been performed to evaluate embryo transfer success rates with prophylactic antibiotics of amoxicillin and clavulanic acid given the day before and the day of transfer ($n = 350$), which showed no difference in clinical pregnancy rates (36% vs 35.5%) [25]. Live birth rates were not reported. Additionally, there is potential harm to the overuse of antibiotics, including microbial resistance, medication side effects, and changes in vaginal and cervical flora [26].

Analgesics and anaesthesia

Analgesics may be used for a variety of reasons, including patient anxiety and vaginismus. No study has been performed on its effects on transfer outcomes, however patient pain has been shown in some studies to affect clinical pregnancy rates. One prospective observational study ($n = 284$) assessing pain experience during embryo transfer showed that patients with a clinical pregnancy had a lower mean pain score (6.4 on a 100 scale) than patients who did not achieve clinical pregnancy (10.3 on a 100 scale), however there were many patients (49.7%) in the non-pregnant group that experienced zero pain [27].

A study of general anaesthesia's effect on the success of embryo transfer was performed in 1988, which showed no benefit with 18% pregnancy rate in the 603 embryo transfers without anaesthesia and a 19% pregnancy rate in the 795 embryo transfers with general anaesthesia (sodium thiopentone and alfentanil), however the practice of IVF has significantly changed since the 1980s [28].

In more recent research, a retrospective case-control study published in 2012 of conscious sedation was performed, but was not adequately powered for significance. It showed live birth rate of embryo transfer with anaesthesia was 21% and without anaesthesia was 40%, however this was not significant with a p-value of 0.09 [29]. A prospective RCT should be performed to clarify the effect of general anaesthesia on transfer.

Gloves worn

The optimal culture and handling of human embryos requires minimizing the exposure of the embryos to toxic equipment and reagents. Gloves worn during transfer, however, do not come into direct contact with the embryo, and only have contact with the portion of the catheter that does not enter the uterine cavity during transfer. There is a theoretical risk of powder from the gloves being transmitted through the air during transfer. However, in a RCT, powdered versus unpowdered gloves did not affect rates of

clinical pregnancy (40.7% vs 39.9%, $n = 712$) [30]. Care should be taken, however, that gloves in general do not come into contact with either the embryo or the transfer end of the catheter during the transfer process.

Transfer techniques

The actual techniques utilized during embryo transfer have both been studied more intensively and have been shown to have a much greater impact on success rates. Great care should be taken to standardize these techniques in order to maximize success.

Time of loading

A prospective observational study was performed on the interval between loading the embryos and discharging the embryos into the uterine cavity ($n = 450$). There was a relationship between the length of time between loading and transfer, with a sharp decline in pregnancy rates when the time interval was more than 120 seconds [31]. When only easy transfers were evaluated, this difference was still observed (19.4% with >120 s vs 38.9–31.6% if <120 s). It is difficult to say what would have caused an easy transfer to last more than 120 seconds. Live birth rate was not evaluated. Nevertheless, taking more than 120 seconds between loading and transfer should be avoided.

Ultrasound guidance

Transabdominal ultrasound was introduced as a method to reduce the risk of endometrial trauma and ensure accurate and appropriate placement of the embryo inside the uterine cavity compared to a blind transfer approach. Many studies have been performed, including a variety of RCTs. A Cochrane meta-analysis looking at a total of 21 included studies ($n = 6218$) showed ultrasound-guided transfer was associated with an increased clinical pregnancy rate compared to blind transfer—OR 1.31 (1.17 to 1.45). Only four trials included live birth rates ($n = 3117$), but showed an OR of 1.53 (1.29 to 1.80), also favouring utilizing transabdominal ultrasound for guidance [32].

Three-dimensional ultrasound guidance

There is some interest in using 3-dimensional (3D) technology in order to guide the catheter tip in the coronal as well as the sagittal plane [33]. Several studies have been done which prove the feasibility of doing so, however there is still a question as to the maximum implantation point within this view [34, 35]. Thus far, a difference in outcomes has not yet been established between the 2D and 3D technique, however non-inferiority has been proven with this technique [35].

Transvaginal ultrasound guidance

Using the transvaginal approach has benefit in that it does not require a full bladder and thus reduces some of the discomfort of an embryo transfer. It may also be useful in patients with difficult visualization abdominally such as those with a retroverted uterus or central adipose tissue distribution [36]. Thus far, success rates have not been shown to differ between the transvaginal and the transabdominal technique [36, 37].

Type of catheter

There are many types of catheters used in embryo transfers, and they can be categorized as “soft” and “firm.” Many studies have been performed comparing the two types [38]; however, firm catheters are almost universally no longer used. A meta-analysis ($n = 4141$) showed a clinical pregnancy OR of 1.39 (1.08–1.79)

with p-value of 0.01 and a live birth rate ($n = 1956$) OR 1.25 (1.02–1.53) [39]. Comparison between different types of soft catheters has been attempted; however, no trials thus far have shown a significant difference in birth rates between soft catheters [38].

Placement of catheter

When placing the embryo inside the uterine cavity, care should be taken for minimal disruption, including avoidance of touching the fundus with the catheter. It is somewhat unclear, however, the exact best placement within the cavity for embryo displacement. Several randomized controlled studies have been done, with the majority showing placement at least 1 cm from the fundus having the highest rates of pregnancy [40–42]. One RCT ($n = 180$) placed embryos at 10 mm, 15 mm, or 20 mm from the fundus, and showed implantation was significantly higher at 15 and 20 mm (31.3% and 33.3%) compared to 10 mm (20.6%; $p < 0.05$) [40].

Removing mucus

If mucus is not removed, it may plug the tip of the catheter, affecting the rate of the embryo expulsion into the uterus, as well as causing embryo retention, damage, or improper placement [43]. There is one randomized controlled prospective trial ($n = 530$), where the cervical canal was cleansed using sterile cotton swabs prior to ET. The live birth rate of the intervention group was 33.6% versus 17.4% in the control group ($p < 0.001$) [44]. A meta-analysis performed, which included eight RCTs involving 1715 patients, however, noted no change in clinical pregnancy rates or live birth rates. These studies, however, were rated as moderate to low quality and had substantial heterogeneity. These studies included removal by aspiration, cotton swab, or by cervical brush [43].

Time interval before withdrawal

There is no evidence to suggest any difference between immediate withdrawal of the embryo catheter after discharge of embryo and a pause before withdrawal. The rationale for delayed withdrawal is waiting for “stabilization” of the uterus to reduce the risk of contractions and expulsion of the embryo from the uterus [45]. An RCT with a 30-second delay ($n = 100$) and a cohort study ($n = 218$) with a 60-second delay showed no change in pregnancy rates based on this timing [45, 46].

Retained embryo

Retention of the embryo in the catheter is an uncommon (1%–8%) [47] but concerning clinical event. As this is a problem that cannot be studied with RCT, retrospective analysis must suffice for evidence. There are many studies which do not suggest any change in rates with immediate reattempt at transfer [48], however they may not be powered adequately to suggest a difference. One well-powered study of 6089 transfer cycles with a retained embryo rate of 1.59% showed a decrease in live birth rate, with 22.68% compared to 37.63% ($p < 0.01$). Additionally, the rate of ectopic pregnancy in the retained embryo group was 12.5% compared to 3.16% without retention ($p = 0.045$) [49]. This suggests that factors aside from simply the retained embryo may impact success in these patients.

Post-transfer techniques

Supine rest after transfer

Bed rest was historically used after transfer; however, like in all of obstetric and gynaecologic care, it has fallen out of favour as a general rule. No duration of bed rest has been shown to demonstrate any benefit, regardless of length. It has been shown to

possibly cause harm, with a recent prospective randomized parallel assignment-controlled trial ($n = 240$) showing a decrease in live birth rates with 10 minutes of rest compared to immediate ambulation, 56.7% versus 41.6% ($p = 0.02$) [50]. This has also been seen in many retrospective cohort trials [51]. Additionally, a meta-analysis of five RCTs, with a total of 1002 women, showed no negative effect of immediate mobilization after an embryo transfer [52].

Intercourse

There are theoretical concerns for uterine contractility after sexual intercourse; however, there are limited studies on its effects after embryo transfer. One RCT ($n = 478$) showed no difference between groups assigned to have intercourse or abstain (23.6% vs 21.2% pregnancy rate at six weeks gestation), albeit the pregnancy rates at baseline were quite low [53].

Conclusion

Embryo transfer can and should be standardized according to our best available data in order to maximize live birth rates. Improvements to technique include using ultrasound guidance for transfer, using soft catheter tips, placing the catheter at least 1 cm from the fundus, minimizing time from loading to transfer completion, and having patients ambulate immediately following transfer. More research, especially well-designed RCTs, should be performed in order to provide up-to-date guidance on all aspects of the transfer process.

References

- Meldrum DR, Chetkowsky R, Steingold KA, de Ziegler D, Cedars MI, Hamilton M. Evolution of a highly successful in vitro fertilization-embryo transfer program. *Fertil Steril*. 1987;48(1):86–93.
- Hearns-Stokes RM, Miller BT, Scott L, Creuss D, Chakraborty PK, Segars JH. Pregnancy rates after embryo transfer depend on the provider at embryo transfer. *Fertil Steril*. 2000;74(1):80–6.
- van Weering HGI, Schats R, McDonnell J, Hompes PGA. Ongoing pregnancy rates in in vitro fertilization are not dependent on the physician performing the embryo transfer. *Fertil Steril*. 2005;83(2):316–20.
- Lee MS, Cardozo ER, Karmon AE, Wright DL, Toth TL. Impact of transfer time on pregnancy outcomes in frozen-embryo transfer cycles. *Fertil Steril*. 2018;109(3):467–72.
- López MJ, García D, Rodríguez A, Colodrán M, Vassena R, Vernaevé V. Individualized embryo transfer training: Timing and performance. *Hum Reprod*. 2014;29(7):1432–7.
- McQueen DB, Robins JC, Zhang J, Feinberg EC. Live birth rates after blastocyst transfers performed by fellows. *Fertil Steril*. 2019;112(3):e145–6.
- McQueen DB, Robins JC, Yeh C, Zhang JX, Feinberg EC. Embryo transfer training in fellowship: National and institutional data. *Fertil Steril*. 2020;114(5):1006–13.
- Segars J, Thomas MA. Validation of a highly realistic embryo transfer simulator and trainer. *Fertil Steril*. 2021;115(4):879–80.
- Ramaiah SD, Ray KA, Reindollar RH. Simulation training for embryo transfer: Findings from the American society for reproductive medicine embryo transfer certificate course. *Fertil Steril*. 2021;115(4):852–9.
- Vitagliano A, Andrisani A, Alviggi C, Vitale SG, Valenti G, Sapia F, et al. Endometrial scratching for infertile women undergoing a first embryo transfer: A systematic review and meta-analysis of published and unpublished data from randomized controlled trials. *Fertil Steril*. 2019;111:734,746.e2.

11. Vitagliano A, Di Spiezo Sardo A, Saccone G, Valentini G, Sapia F, Kamath MS, et al. Endometrial scratch injury for women with one or more previous failed embryo transfers: A systematic review and meta-analysis of randomized controlled trials. *Fertil Steril.* 2018;110:687–702.e2.
12. Lensen SF, Lensen SF, Armstrong S, Gibreel A, Nastri CO, Raine-Fenning N, et al. Endometrial injury in women undergoing in vitro fertilisation (IVF). *Cochrane library* 2021;2021:CD009517.
13. Morin SJ, Frattarelli JL, Franasiak JM, Juneau CR, Scott RT. Laser acupuncture before and after embryo transfer improves in vitro fertilization outcomes: A four-armed randomized controlled trial. *Med Acupunct* 2017;29:56–65.
14. Zheng, Cui Hong, Huang, Guang Ying, Zhang, Ming Min, Wang, Wei. Effects of acupuncture on pregnancy rates in women undergoing in vitro fertilization: A systematic review and meta-analysis. *Fertil Steril.* 2012;97(3):599–611.
15. Zhang R, Feng X, Guan Q, Cui W, Zheng Y, Sun W, et al. Increase of success rate for women undergoing embryo transfer by transcutaneous electrical acupoint stimulation: A prospective randomized placebo-controlled study. *Fertil Steril.* 2011;96(4):912–6.
16. Okhowat J, Murtinger M, Schuff M, Wogatzky J, Spitzer D, Vanderzwalmen P. Massage therapy improves in vitro fertilization outcome in patients undergoing blastocyst transfer in a cryo-cycle. *Altern Ther Health Med.* 2015;21(2):16–22.
17. Chung CHS, Wong AWY, Chan CPS, Saravelos SH, Kong GWS, Cheung LP, et al. The changing pattern of uterine contractions before and after fresh embryo transfer and its relation to clinical outcome. *Reprod Biomed Online.* 2016;34(3):240–7.
18. Griesinger G, Blockeel C, Pierzynski P, Tournaye H, Višňová H, Humberstone A, et al. Effect of the oxytocin receptor antagonist nolasiban on pregnancy rates in women undergoing embryo transfer following IVF: Analysis of three randomised clinical trials. *Hum Reprod.* 2021 Mar 18;36(4):1007–20.
19. Neumann K, Griesinger G. Is the administration of an oxytocin receptor antagonist around the time of embryo transfer associated with in-vitro fertilization treatment success? A systematic review and meta-analysis. *Reprod Biomed Online.* 2021;43(6):983–94.
20. He Y, Wu H, He, Xiaojin, Xing Q, Zhou P, Cao, Yunxia, et al. Administration of atosiban in patients with endometriosis undergoing frozen-thawed embryo transfer: A prospective, randomized study. *Fertil Steril.* 2016;106(2):416–22.
21. Salim R, Ben-Shlomo I, Colodner R, Keness Y, Shalev E. Bacterial colonization of the uterine cervix and success rate in assisted reproduction: Results of a prospective survey. *Hum Reprod.* 2002;17(2):337–40.
22. Egbase P, Udo E, Al-Sharhan M, Grudzinskas J. Prophylactic antibiotics and endocervical microbial inoculation of the endometrium at embryo transfer. *Lancet.* 1999;354(9179):651–2.
23. Fanchin R, Harmas A, Benaoudia F, Lundkvist U, Olivennes F, Frydman R. Microbial flora of the cervix assessed at the time of embryo transfer adversely affects in vitro fertilization outcome. *Fertil Steril.* 1998;70(5):866–70.
24. Kaye L, Bartels C, Bartolucci A, Engmann L, Nulsen J, Benadiva C. Old habits die hard: Retrospective analysis of outcomes with use of corticosteroids and antibiotics before embryo transfer. *Fertil Steril.* 2017;107(6):1336–40.
25. Brook N, Khalaf Y, Coomarasamy A, Edgeworth J, Braude P. A randomized controlled trial of prophylactic antibiotics (co-amoxiclav) prior to embryo transfer. *Hum Reprod.* 2006 Jul 10;21(11):2911–15.
26. Bonus ML, Boots C, Bernardi LA. Antibiotics before frozen embryo transfer: Treating the patient or the physician? *Fertil Steril.* 2021;115(5):1162–3.
27. Saravelos SH, Wong AW, Kong GW, Huang J, Klitzman R, Li T. Pain during embryo transfer is independently associated with clinical pregnancy in fresh/frozen assisted reproductive technology cycles. *J Obstet Gynaecol Res.* 2016;42(6):684–93.
28. van der Ven H, Diedrich K, Al-Hasani S, Pless V, Krebs D. The effect of general anaesthesia on the success of embryo transfer following human in-vitro fertilization. *Hum Reprod.* 1988;3(suppl 2):81–83.
29. Meyer L, Lekovic J, Reichman D, Chung P, Rosenwaks Z. The effect of anesthesia during embryo transfer on in vitro fertilization outcome. *Fertil Steril.* 2013;100(3):S526–7.
30. Hannoun A, Zreik T, Ghaziri G, Abu Musa A, Awwad J. Effect of powdered gloves, worn at the time of embryo transfer, on the pregnancy outcome of IVF cycles. *J Assist Reprod Genet.* 2009;26(1):25–7.
31. Matorras R, Mendoza R, Expósito A, Rodriguez-Escudero FJ. Influence of the time interval between embryo catheter loading and discharging on the success of IVF. *Hum Reprod.* 2004;19:2027–30.
32. Brown J, Buckingham K, Buckett W, Abou-Setta AM. Ultrasound versus ‘clinical touch’ for catheter guidance during embryo transfer in women. *Cochrane Database Syst Rev.* 2016 Mar 17;3:CD006107.
33. Baba K, Ishihara O, Hayashi N, Saitoh M, Taya J, Kinoshita K. Three-dimensional ultrasound in embryo transfer. *Ultrasound Obstet Gynecol.* 2000;16(4):372–3.
34. Saravelos SH, Kong GWS, Chung JPW, Mak JSM, Chung CHS, Cheung LP, et al. A prospective randomized controlled trial of 3D versus 2D ultrasound-guided embryo transfer in women undergoing ART treatment. *Hum Reprod.* 2016 Oct 1;31(10):2255–60.
35. Gergely RZ, DeUgarte CM, Danzer H, Surrey M, Hill D, DeCherney AH. Three dimensional/four dimensional ultrasound-guided embryo transfer using the maximal implantation potential point. *Fertil Steril.* 2005;84(2):500–3.
36. Revelli A, Rovei V, Dalmasso P, Gennarelli G, Racca C, Evangelista F, et al. Large randomized trial comparing transabdominal ultrasound-guided embryo transfer with a technique based on uterine length measurement before embryo transfer. *Ultrasound Obstet Gynecol.* 2016 Sep;48(3):289–95.
37. Karavani G, Ben-Meir A, Shufaro, Y, Hyman JH, Revel A. Transvaginal ultrasound to guide embryo transfer: A randomized controlled trial. *Fertil Steril.* 2017;107(5):1159–65.
38. Buckett WM. A review and meta-analysis of prospective trials comparing different catheters used for embryo transfer. *Fertil Steril.* 2006;85(3):728–34.
39. Abou-Setta AM, Al-Inany HG, Mansour RT, Serour GI, Aboulghar MA. Soft versus firm embryo transfer catheters for assisted reproduction: A systematic review and meta-analysis. *Hum Reprod.* 2005;20(11):3114–21.
40. Coroleu B, Barri PN, Carreras O, Martínez F, Parriego M, Herreter L, et al. The influence of the depth of embryo replacement into the uterine cavity on implantation rates after IVF: A controlled, ultrasound-guided study. *Hum Reprod.* 2002 Feb;17(2):341–6.
41. Tiras B, Polat M, Korucuoglu U, Zeyneloglu HB, Yarali H. Impact of embryo replacement depth on in vitro fertilization and embryo transfer outcomes. *Fertil Steril.* 2010;94(4):1341–5.
42. Kwon H, Choi D, Kim E. Absolute position versus relative position in embryo transfer: A randomized controlled trial. *Reprod Biol Endocrinol.* 2015 Jul 29;13(1):78.
43. Craciunas L, Tsampras N, Fitzgerald C. Cervical mucus removal before embryo transfer in women undergoing in vitro fertilization/intracytoplasmic sperm injection: A systematic review and meta-analysis of randomized controlled trials. *Fertil Steril.* 2014;101(5):1302–7.e6.
44. Moini A, Kiani K, Bahmanabadi A, Akhoond M, Akhlaghi A. Improvement in pregnancy rate by removal of cervical discharge prior to embryo transfer in ICSI cycles: A randomised clinical trial. *Aust NZ J Obstet Gynaecol.* 2011;51(4):315–20.
45. Martínez F, Coroleu B, Parriego M, Carreras O, Belil I, Parera N, et al. Ultrasound-guided embryo transfer: Immediate withdrawal of the catheter versus a 30 second wait. *Hum Reprod.* 2001 May;16(5):871–4.

46. Sroga JM, Montville, Christopher P, Aubuchon M, Williams DB, Thomas MA. Effect of delayed versus immediate embryo transfer catheter removal on pregnancy outcomes during fresh cycles. *Fertil Steril.* 2010;93(6):2088–90.
47. Lee H, Seifer DB, Shelden RM. Impact of retained embryos on the outcome of assisted reproductive technologies. *Fertil Steril* 2004;82:334–7.
48. Oraif, Ayman, Hollet-Caines, Jackie, Feyles, Valter, Rebel, Maggie, Abduljabar H. Do multiple attempts at embryo transfer affect clinical pregnancy rates? *J Obstet Gynaecol Canada* 2014;36:406–7.
49. Xu J, Yin M, Chen Z, Yang L, Ye D, Sun L. Embryo retention significantly decreases clinical pregnancy rate and live birth rate: a matched retrospective cohort study. *Fertil Steril* 2020;114: 787–91.
50. Gaikwad S, Garrido N, Cobo A, Pellicer A, Remohi J. Bed rest after embryo transfer negatively affects in vitro fertilization: A randomized controlled clinical trial. *Fertil Steril.* 2013;100(3): 729–35.e2.
51. Sharif K, Afnan M, Lashen H, Elgendi M, Morgan C, Sinclair L. Is bed rest following embryo transfer necessary? *Fertil Steril.* 1998;69(3):478–81.
52. Cozzolino M, Troiano G, Esencan E. Bed rest after an embryo transfer: A systematic review and meta-analysis. *Arch Gynecol Obstet.* 2019;300(5):1121–30.
53. Tremellen KP, Valbuena D, Landeras J, Ballesteros A, Martinez J, Mendoza S, et al. The effect of intercourse on pregnancy rates during assisted human reproduction. *Hum Reprod.* 2000 Dec;15(12):2653–8.

CYCLE REGIMES FOR FROZEN-THAWED EMBRYO TRANSFER

Jane Reavey and Tim Child

In the past decades, there has been a significant rise in the number of frozen embryo transfer (FET) cycles. In the United Kingdom, frozen cycles continue to increase annually and now account for more than 41% of all assisted conception cycles [1] with similar increases globally [2]. This increase is a result of improvements in embryology techniques and live birth rates (LBR), which are now similar [3] or higher [3, 4] than those of a fresh IVF cycle.

Ovarian stimulation commonly results in the generation of more embryos than are necessary for fresh embryo transfer. Therefore, cryopreservation and subsequent replacement of a frozen-thawed embryo is an integral part of assisted reproductive technology (ART) programs. FET cycles have played a key role in enabling a reduction in multiple pregnancy rates through elective single-embryo transfer (eSET) in a fresh cycle followed by a subsequent FET if pregnancy does not occur.

Indications for FET cycles have also broadened. Planned “freeze-all” cycles (in which all suitable embryos are frozen) reduce the risk of ovarian hyperstimulation syndrome (OHSS), allow time for pre-implantation genetic testing, and facilitate fertility preservation.

This chapter will outline the process of FET, review the different protocols for endometrial preparation and luteal support, and also consider pregnancy outcomes from FET cycles.

FET protocols

It is vital that a frozen-thawed embryo is replaced during the window of endometrial receptivity and that there is synchronization between an embryo and endometrial development. A number of different protocols have been developed to achieve this: (i) replacement during a natural ovulatory cycle; (ii) hormone (oestrogen and progesterone) replacement cycles (with or without prior or synchronous pituitary downregulation); and (iii) ovulation induction cycles.

As endometrial receptivity may be negatively affected by the supra-physiologic hormone levels associated with ovarian stimulation [5, 6], it was suggested that there may be an advantage to performing elective embryo freezing and replacement for all women in preference to fresh embryo transfer [7]. However, a number of randomized controlled trials (RCTs), including a recent UK multicentre trial [3, 8, 9], show no differences in clinical pregnancy, live birth, or miscarriage rates with an elective freeze-all policy. When specifically considering women at high risk of OHSS, trials have suggested that an elective freeze-all strategy in this group does increase live birth per embryo transfer [10, 11]. Therefore, at present, an individualized approach should be performed, with freeze-all being reserved for specific indications, including high-responding women.

Despite the worldwide increase in FET cycles, the best protocol for endometrial preparation in ovulatory women remains unclear. Data from a small number of RCTs and meta-analyses comparing

natural versus hormone replacement protocols have shown little difference in pregnancy and LBR between each method [12–15].

Natural FET cycles

In natural FET cycles, embryo transfer is usually timed using a combination of ultrasound monitoring to confirm follicular development and urinary or serum detection of the luteinizing hormone (LH) surge. The major advantage to replacement in a natural FET cycle is that no medication is required, which reduces costs. However, the rate of cycle cancellation is higher in a natural FET cycle (~9%) compared to a hormone replacement cycle (~2%) [15]. There will also be a significant proportion of women for whom this approach is not suitable, such as women with anovulatory polycystic ovary syndrome (PCOS).

Some clinics advocate the use of a “modified natural cycle” where human chorionic gonadotropin (hCG) is used to trigger ovulation and aid in the timing of embryo replacement. Studies investigating this are limited with conflicting results. Three small RCTs have compared the use of an hCG trigger to ultrasound with LH monitoring. One study was terminated early after interim analysis because of a significantly lower pregnancy rate in those randomized to an hCG trigger [16]. However, further RCTs showed that the use of an hCG trigger (compared to ultrasound with LH monitoring) resulted in no difference in clinical pregnancy rates (CPR) [17, 18].

The use of supplementary progesterone in the luteal phase has also been studied in four RCTs with conflicting results. Two RCTs found no improvement in CPR when intramuscular [19] or vaginal progesterone [20] was given in the luteal phase. Two further RCTs [21, 22] and a meta-analysis [23] have shown a significant increase in LBR in women receiving vaginal progesterone in the luteal phase. A further multicentre, large RCT comparing the use of vaginal progesterone in the luteal phase to no luteal support is ongoing (NCT04725864). Therefore, to date, there is no consensus on luteal phase support in natural FET cycles and future studies (as discussed later) may focus on a more individualized approach based on progesterone levels at the time of embryo transfer.

Hormone replacement cycles

Hormone replacement, or programmed, cycles were originally developed for women with ovarian failure having FET in the context of an oocyte donation programme. However, they are now the most widely used protocol for endometrial preparation [24], in part due to the increased flexibility as to the timing of embryo transfer that may suit both the patient and the clinic (e.g. the avoidance of weekend thawing and transfers) and reduced need for cycle monitoring. A number of different protocols exist. First, ovarian downregulation can be achieved by the use of a gonadotropin-releasing hormone (GnRH) agonist for two to three weeks, after which oestrogen and then progesterone is used. A simpler regime commencing oestrogen on day 2 or 3 of the cycle (which prevents follicular recruitment) with the addition of progesterone later, with or without the use of a GnRH antagonist, is also commonly followed.

Only a small number of RCTs have studied the use of GnRHa downregulation and subsequent hormone replacement compared to using oestrogen and progesterone alone. A Cochrane meta-analysis of these RCTs showed no difference in pregnancy rate, cycle cancellation, endometrial thickness, or miscarriage rate [14]. Notably, the only study to report LBR found a statistically significant increase in the group that underwent downregulation [25]. In that study, ovarian activity was not monitored, with the risk of “escape ovulation,” and the authors concluded that in medicated cycles, when embryo replacement was determined by endometrial thickness alone, ovarian activity should either be monitored or suppressed. An RCT comparing medicated FET with and without a GnRH antagonist is ongoing ([clinicaltrials.gov](#), NCT03763786).

Hormone preparations in hormone replacement FET cycles

A number of preparations of both oestrogen and progesterone have been used in FET cycles. Commonly, oestrogen is administered in tablet form or transdermal patches. One RCT comparing the use of oestradiol patches versus oral oestradiol in FET cycles found no difference in CPR [26]. Generally, around 14 days of oestrogen supplementation is given, corresponding with the length of a natural follicular stage. However, flexibility within this does not appear to compromise endometrial receptivity, and a recent RCT has shown no difference in pregnancy outcomes comparing 7–14 days of oestrogen supplementation prior to FET [27]. Once an adequate endometrial thickness is achieved, progesterone is administered to initiate secretory changes within the endometrium. Progesterone (micronized or synthetic) may be given as a tablet, pessary (rectal or transvaginal), or by subcutaneous or intramuscular injection. Although the majority of clinics use vaginal progesterone [28], which avoids the potential complications of pain and abscess formation from intramuscular injections, good evidence on the optimal route or dose is lacking. An RCT comparing progesterone preparations in non-downregulated medicated cycles found no difference in pregnancy rates when comparing vaginal micronized tablets and vaginal progesterone gel [29]. Although a systematic review observed a trend in improved CPR with vaginal compared to intramuscular progesterone, no difference in pregnancy rates was seen in an RCT comparing these two routes of administration [30]. A more recent RCT contradicts these data and found that vaginal progesterone replacement alone resulted in lower LBR compared to either intramuscular progesterone or vaginal progesterone supplemented by intramuscular progesterone every third day [31]. However, the dose of vaginal progesterone (400 mg daily) was significantly less than that commonly used in Europe and elsewhere (600–800 mg daily).

Further research, rather than focusing on fixed progesterone one administration protocols, may consider a more individualized approach. This is due to growing evidence that a threshold serum concentration of progesterone may be required for optimal embryo implantation in FET. Currently, the majority of UK clinics do not measure serum progesterone in FET [24]. However, a recently published systematic review [32] found that women with a serum progesterone level <10 ng/mL at the time of embryo transfer in FET cycles had a higher risk of miscarriage and lower clinical pregnancy and LBR. A recent retrospective study [33] found that giving additional progesterone supplementation to women with low serum progesterone levels resulted in LBRs similar to women with adequate progesterone levels. However,

other studies have failed to show any differences [34]. Therefore, it remains unclear whether additional “rescue” progesterone in women with low serum levels can improve pregnancy outcomes, and RCTs are required to investigate this further.

Embryo transfer is normally on the fifth or sixth day of progesterone administration for blastocysts [35, 36]. RCTs investigating a longer duration (seven days) of progesterone administration have not shown any benefit. One RCT comparing seven to five days of progesterone prior to FET found no difference in LBR or early pregnancy loss [37]. Another RCT comparing seven days of progesterone to six days showed no difference in CPR [38]. Although there are no studies regarding the optimal duration of continued progesterone support in pregnancy following FET, most clinics advise patients to continue progesterone treatment for 8–12 weeks, by which time placental progesterone production is adequate.

It has been hypothesized that hCG may have a beneficial effect on the secretory endometrium, stimulating cytokines and proteins that are important to implantation. However, two RCTs of hCG supplementation versus no treatment in non-downregulated, hormonally induced cycles, showed no significant difference in the CPR [39, 40]. Two RCTs have investigated whether glucocorticoids improve implantation or CPR in FET; however, neither study showed a benefit [41, 42]. One RCT has looked at the use of sildenafil citrate in artificial FET cycles. Although the endometrial thickness and presence of a triple line were significantly higher in the treatment group, this did not translate to higher implantation or CPRs [43].

Stimulation regimes for FET

An alternative approach to endometrial preparation for FET cycles is to use low-dose ovarian stimulation with gonadotrophin injections, clomiphene citrate, or letrozole. As ovulation induction cycles require increased monitoring, are relatively expensive, and do not have the advantage of flexibility with regard to the timing of embryo replacement, few centres use this regime. There is also no consensus on the optimal protocol for ovarian stimulation.

A stimulated regime can be considered in women with anovulatory cycles or for women with regular cycles who wish to avoid natural FET. However, in ovulatory women, stimulated regimes do not appear to provide any benefit compared to FET in a natural cycle. A retrospective study [44] showed no difference in LBRs when comparing natural cycle to clomiphene citrate stimulation. Mild ovarian stimulation with low-dose human menopausal gonadotropin was also compared to natural cycle in an RCT of 410 ovulatory women. There were no differences in endometrial thickness, implantation rate, or LBR between the two groups [45].

However, a recent systematic review has shown advantages with stimulated cycles using letrozole versus hormone replacement cycles in women with anovulatory PCOS. Higher LBRs, lower miscarriage rates, and reduced rates of pre-eclampsia were seen in the letrozole group [46]. A further meta-analysis supported this reduced miscarriage rate in letrozole-stimulated cycles [47].

Endometrial thickness and quality in FET cycles

Several studies have failed to identify differences in endometrial thickness and morphology between conception and non-conception cycles in both natural and medicated cycles [48, 49]. However, a large retrospective study of medicated (non-downregulated)

FET cycles found that implantation and pregnancy rates were significantly lower when the endometrial thickness was less than 7 mm or greater than 14 mm [50]. A retrospective analysis of more than 18,000 FET cycles showed a reduction in clinical pregnancy and LBR with each millimetre decrease in endometrial thickness below 7 mm [51]. However, in some women, this optimal endometrial thickness may not be achievable, and the study highlighted that acceptable LBR (15%–21%) were still seen in women with an endometrial thickness of 4–6 mm. Although in fresh *in vitro* fertilization (IVF) cycles a triple line is associated with an increased CPR, in FET cycles, no such association has been identified [49, 52]. Endometrial compaction (a decrease in endometrial thickness at the time of warmed blastocyst transfer) has been reported as a favourable predictor for pregnancy in FET [53, 54], though other studies do not support this [55, 56]. There has also been growth in the development of endometrial receptivity tests where analysis of gene expression in endometrial samples taken at the time of potential embryo transfer can predict either a receptive or non-receptive endometrium. However, more evidence is needed to evaluate the accuracy of these tests and their potential impact on pregnancy outcomes [57].

The developmental stage and quality of the embryo at the time of freezing

Embryo cryopreservation has been successfully achieved at the pronuclear (day 1), cleavage (days 2–4), and blastocyst (days 5–6) stages. However, studies have now repeatedly demonstrated higher LBR when embryos were cryopreserved at the blastocyst stage compared to earlier stages [58, 59]. Improved embryology techniques with a shift from slow-freezing to vitrification have radically changed the potential of blastocyst cryopreservation. Post-thaw survival of vitrified blastocysts is in the range of 80%–100% [60, 61]. A survey of UK fertility clinics showed that the vast majority of responding clinics now favour cryopreservation at the blastocyst stage [24].

Studies have shown conflicting results as to whether the rate of blastocyst formation affects the treatment outcome. A meta-analysis has shown a significant increase in the CPR and LBR when day 5 rather than day 6 frozen-thawed blastocysts are transferred. However, this difference was no longer seen where the day 5 and day 6 embryos had the same morphological quality [62].

Zona pellucida breaching

It is thought that the process of cryopreservation may cause hardening of the zona pellucida [63], and therefore assisted hatching may be beneficial in FET cycles by improving embryo implantation. However, there is no evidence currently to support this. One RCT [64] found no difference in clinical pregnancy or LBR when assisted hatching was performed compared to no intervention.

Refreezing of thawed embryos

There are a number of reports of successful live births after the transfer of embryos that have been frozen and thawed more than once [48, 65, 66]. Perinatal outcomes appear to be reassuring [67]. Although the routine use of multiple freeze thaws is not recommended because of the potential stress of cryopreservation, it may be of value in particular circumstances. A recent retrospective case-control study [66] comparing the transfer of twice to

single cryopreserved embryos found similar clinical pregnancy, live birth, and miscarriage rates.

Maternal and perinatal outcomes of FET cycles

The safety of embryo cryopreservation has been questioned. Concerns have been raised regarding its effects on embryonic gene expression and metabolism, as well as the potential negative effects of cryoprotectants [68]. However, a register-based study showed no significant difference in the physical health outcomes at three years of age between children born from fresh compared to frozen cycles [69]. Two meta-analyses [70, 71] comparing fresh versus frozen embryo cycles have shown that babies conceived through FET actually have a lower risk of preterm delivery, small for gestational age, and low birthweight. In addition, no difference in the risk of congenital anomalies, antepartum haemorrhage, perinatal mortality, or neonatal unit admission has been found [70–72]. However, a number of cohort studies and meta-analyses have reported an increased risk of large for gestational-age babies with FET compared to fresh IVF [71, 73, 74]. This has implications not just for birth (with an increased risk of shoulder dystocia) but also health and diseases later in life.

A further concern is an association between FET and increased risk of hypertensive disorders of pregnancy (gestational hypertension, pre-eclampsia, and eclampsia). This has been shown both in meta-analyses and a recent RCT comparing fresh versus frozen cycles [4, 70, 71]. Interestingly, there is emerging evidence that this association is actually specific to hormone replacement FETs. A recent retrospective cohort study of patients undergoing natural cycle FET compared to hormone replacement FET [75] found that the risk of developing pre-eclampsia was significantly associated with hormone replacement FET. This finding of an increased risk of gestational hypertensive disorders in hormone replacement compared to natural cycle FET has been shown in previous cohort studies also [76, 77], with the hypothesis that in hormone replacement cycles the absence of the corpus luteum, a source of important vasoactive substances, may lead to an increased risk of abnormal maternal cardiovascular adaptation to pregnancy [78]. In view of this, there is ongoing debate about the application of hormone replacement cycles to ovulatory women [77]. Further research from RCTs specifying the endometrial protocols used is required to explore this further. An RCT [79] comparing pre-eclampsia rates with natural compared to hormone replacement cycles is currently ongoing (NCT04551807). Until then, these potential risks are important to consider when counselling and consenting women for FET and also considering options for endometrial preparation.

Conclusions

FET cycles continue to rise worldwide due to improved embryology techniques and birth rates. Evidence to date supports elective freeze-all cycles for specific indications only. Overall, there are no conclusions regarding the best method of endometrial preparation. However, recent evidence on increased rates of pre-eclampsia, specifically in hormone replacement cycles, may influence the choice of protocol and change future practice. Further studies investigating progesterone levels in the luteal phase will also help to clarify whether additional progesterone replacement may improve pregnancy outcomes for millions of women undergoing FET worldwide.

References

1. HFEA. *Human Fertilisation and Embryology Authority: Fertility Treatment 2019: Trends and Figures*. London: HFEA, 2021.
2. Chambers GM, et al. International committee for monitoring assisted reproductive technologies world report: assisted reproductive technology, 2014†. *Hum Reprod*. 2021;36(11):2921–34.
3. Vuong LN, et al. IVF transfer of fresh or frozen embryos in women without polycystic ovaries. *N Engl J Med*. 2018;378(2):137–47.
4. Wei D, et al. Frozen versus fresh single blastocyst transfer in ovulatory women: A multicentre, randomised controlled trial. *Lancet*. 2019;393(10178):1310–8.
5. Haouzi D, et al. Gene Expression profile of human endometrial receptivity: Comparison between natural and stimulated cycles for the same patients. *Hum Reprod*. 2009;24(6):1436–45.
6. Haouzi D, et al. Controlled ovarian hyperstimulation for in vitro fertilization alters endometrial receptivity in humans: Protocol effects. *Biol Reprod*. 2010;82(4):679–86.
7. Roque M, et al. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: A systematic review and meta-analysis. *Fertil Steril*. 2013;99(1):156–62.
8. Shi Y, et al. Transfer of fresh versus frozen embryos in ovulatory women. *N Engl J Med*. 2018;378(2):126–36.
9. Maheshwari A, et al. Elective freezing of embryos versus fresh embryo transfer in IVF: A multicentre randomized controlled trial in the UK (E-freeze). *Hum Reprod*. 2022;37(3):476–87.
10. Aflatoonian A, et al. Fresh versus frozen embryo transfer after gonadotropin-releasing hormone agonist trigger in gonadotropin-releasing hormone antagonist cycles among high responder women: A randomized, multi-center study. *Int J Reprod Biomed*. 2018;16(1):9–18.
11. Chen ZJ, et al. Fresh versus frozen embryos for infertility in the polycystic ovary syndrome. *N Engl J Med*. 2016;375(6):523–33.
12. Groenewoud ER, et al. What is the optimal means of preparing the endometrium in frozen-thawed embryo transfer cycles? A systematic review and meta-analysis. *Hum Reprod Update*. 2013;19(5):458–70.
13. Groenewoud ER, et al. A randomized controlled, non-inferiority trial of modified natural versus artificial cycle for cryo-thawed embryo transfer. *Hum Reprod*. 2016;31(7):1483–92.
14. Ghobara T, Gelbaya TA, Ayeleke RO. Cycle regimens for frozen-thawed embryo transfer. *Cochrane Database Syst Rev*. 2017;7(7): Cd003414.
15. Mounce G, et al. Randomized, controlled pilot trial of natural versus hormone replacement therapy cycles in frozen embryo replacement in vitro fertilization. *Fertil Steril*. 2015;104(4):915–20.e1.
16. Fatemi HM, et al. Cryopreserved-thawed human embryo transfer: Spontaneous natural cycle is superior to human chorionic gonadotropin-induced natural cycle. *Fertil Steril*. 2010;94(6):2054–8.
17. Weissman A, et al. Spontaneous ovulation versus HCG triggering for timing natural-cycle frozen-thawed embryo transfer: A randomized study. *Reprod Biomed Online*. 2011;23(4):484–9.
18. Mackens S, et al. To trigger or not to trigger ovulation in A natural cycle for frozen embryo transfer: A randomized controlled trial. *Hum Reprod*. 2020;35(5):1073–81.
19. Eftekhar M, Rahsepar M, Rahmani E. Effect of progesterone supplementation on natural frozen-thawed embryo transfer cycles: A randomized controlled trial. *Int J Fertil Steril*. 2013;7(1): 13–20.
20. Horowitz E, et al. A randomized controlled trial of vaginal progesterone for luteal phase support in modified natural cycle - frozen embryo transfer. *Gynecol Endocrinol*. 2021;37(9):792–7.
21. Bjuresten K, et al. Luteal phase progesterone increases live birth rate after frozen embryo transfer. *Fertil Steril*. 2011;95(2):534–7.
22. Wånggren K, et al. Progesterone supplementation in natural cycles improves live birth rates after embryo transfer of frozen-thawed embryos-a randomized controlled trial. *Hum Reprod*. 2022;37(10):2366–74.
23. Mizrahi Y, et al. Should women receive luteal support following natural cycle frozen embryo transfer? A systematic review and meta-analysis. *Hum Reprod Update*. 2021;27(4):643–50.
24. Noble M, Child T. A UK-wide cross-sectional survey of practice exploring current trends in endometrial preparation for frozen-thawed embryo replacement. *Hum Fertil (Camb)*. 2020;25(2): 283–90.
25. El-Toukhy T, et al. Pituitary suppression in ultrasound-monitored frozen embryo replacement cycles. A randomised study. *Hum Reprod*. 2004;19(4):874–9.
26. Davar R, et al. A comparison of the effects of transdermal estradiol and estradiol valerate on endometrial receptivity in frozen-thawed embryo transfer cycles: A randomized clinical trial. *J Reprod Infertil*. 2016;17(2):97–103.
27. Jiang WJ, Song JY, Sun ZG. Short (seven days) versus standard (fourteen days) oestrogen administration in a programmed frozen embryo transfer cycle: A retrospective cohort study. *J Ovarian Res*. 2022;15(1):36.
28. Shoham G, Leong M, Weissman A. A 10-year follow-up on the practice of luteal phase support using worldwide web-based surveys. *Reprod Biol Endocrinol*. 2021;19(1):15.
29. Lan VT, et al. Progesterone supplementation during cryopreserved embryo transfer cycles: Efficacy and convenience of two vaginal formulations. *Reprod Biomed Online*. 2008;17(3):318–23.
30. Lightman A, Kol S, Itskovitz-Eldor J. A prospective randomized study comparing intramuscular with intravaginal natural progesterone in programmed thaw cycles. *Hum Reprod*. 1999;14(10):2596–9.
31. Devine K, et al. Intramuscular progesterone optimizes live birth from programmed frozen embryo transfer: A randomized clinical trial. *Fertil Steril*. 2021;116(3):633–43.
32. Melo P, et al. Serum luteal phase progesterone in women undergoing frozen embryo transfer in assisted conception: A systematic review and meta-analysis. *Fertil Steril*. 2021;116(6):1534–56.
33. Labarta E, et al. Individualized luteal phase support normalizes live birth rate in women with low progesterone levels on the day of embryo transfer in artificial endometrial preparation cycles. *Fertil Steril*. 2022;117(1):96–103.
34. Volovsky M, et al. Do serum progesterone levels on day of embryo transfer influence pregnancy outcomes in artificial frozen-thaw cycles? *J Assist Reprod Genet*. 2020;37(5):1129–35.
35. Gluovsky D, et al. Endometrial preparation for women undergoing embryo transfer with frozen embryos or embryos derived from donor oocytes. *Cochrane Database Syst Rev*. 2010(1): Cd006359.
36. Mackens S, et al. Frozen embryo transfer: A review on the optimal endometrial preparation and timing. *Hum Reprod*. 2017;32(11): 2234–42.
37. van de Vijver A, et al. Vitrified-warmed blastocyst transfer on the 5th or 7th day of progesterone supplementation in an artificial cycle: A randomised controlled trial. *Gynecol Endocrinol*. 2017;33(10):783–6.
38. Ding J, Rana N, Dmowski WP. Length of progesterone treatment before transfer and implantation rate of frozen-thawed blastocysts. *Fertil Steril*. 2007;88(supplement 1):S330–1.
39. Ben-Meir A, et al. The benefit of human chorionic gonadotropin supplementation throughout the secretory phase of frozen-thawed embryo transfer cycles. *Fertil Steril*. 2010;93(2):351–4.
40. Shiotani M, et al. Is human chorionic gonadotropin supplementation beneficial for frozen and thawed embryo transfer in estrogen/progesterone replacement cycles? A randomized clinical trial. *Reprod Med Biol*. 2017;16(2):166–9.
41. Bider D, et al. Glucocorticoid administration during transfer of frozen-thawed embryos: A prospective, randomized study. *Fertil Steril*. 1996;66(1):154–6.
42. Moffitt D, et al. Low-dose glucocorticoids after in vitro fertilization and embryo transfer have no significant effect on pregnancy rate. *Fertil Steril*. 1995;63(3):571–7.

43. Firouzabadi R, et al. Effect of sildenafil citrate on endometrial preparation and outcome of frozen-thawed embryo transfer cycles: A randomized clinical trial. *Iran J Reprod Med.* 2013;11(2):151–8.
44. Kyrou D, et al. Transfer of cryopreserved - thawed embryos in hCG induced natural or clomiphene citrate cycles yields similar live birth rates in normo-ovulatory women. *J Assist Reprod Genet.* 2010;27(12):683–9.
45. Peeraer K, et al. Frozen-thawed embryo transfer in A natural or mildly hormonally stimulated cycle in women with regular ovulatory cycles: A RCT. *Hum Reprod.* 2015;30(11):2552–62.
46. Zhang Y, et al. Systematic review update and meta-analysis of randomized and non-randomized controlled trials of ovarian stimulation versus artificial cycle for endometrial preparation prior to frozen embryo transfer in women with polycystic ovary syndrome. *Reprod Biol Endocrinol.* 2022;20(1):62.
47. Zeng MF, Zhou X, Duan JL. Stimulated cycle versus artificial cycle for frozen embryo transfer in patients with polycystic ovary syndrome: A meta-analysis. *Gynecol Endocrinol.* 2021;37(4):294–9.
48. Check JH, et al. Pregnancy rates following the exclusive transfer of twice frozen twice thawed embryos using a modified slow cool cryopreservation technique. *Clin Exp Obstet Gynecol.* 2013;40(1):20–1.
49. Coulam CB, et al. Ultrasonographic predictors of implantation after assisted reproduction. *Fertil Steril.* 1994;62(5):1004–10.
50. El-Toukhy T, et al. The relationship between endometrial thickness and outcome of medicated frozen embryo replacement cycles. *Fertil Steril.* 2008;89(4):832–9.
51. Liu KE, et al. The impact of a thin endometrial lining on fresh And frozen-thaw IVF outcomes: An Analysis of over 40 000 embryo transfers. *Hum Reprod.* 2018;33(10):1883–8.
52. Ng EH, et al. The role of endometrial and subendometrial vascular-ity measured by three-dimensional power Doppler ultrasound in the prediction of pregnancy during frozen-thawed embryo trans-fer cycles. *Hum Reprod.* 2006;21(6):1612–7.
53. Haas J, et al. Endometrial compaction (decreased thickness) in response to progesterone results in optimal pregnancy outcome in frozen-thawed embryo transfers. *Fertil Steril.* 2019;112(3):503–9.e1.
54. Zilberman E, et al. Endometrial compaction before frozen euploid embryo transfer improves ongoing pregnancy rates. *Fertil Steril.* 2020;113(5):990–5.
55. Ye J, et al. Effect of endometrial thickness change in response to progesterone administration on pregnancy outcomes in frozen-thawed embryo transfer: Analysis of 4465 cycles. *Front Endocrinol (Lausanne).* 2020;11:546232.
56. Huang J, et al. Significance of endometrial thickness change after human chorionic gonadotrophin triggering in modified natu-ral cycles for frozen-thawed embryo transfer. *Ann Transl Med.* 2020;8(23):1590.
57. Lensen S, et al. The role of timing in frozen embryo transfer. *Fertil Steril.* 2022;118(5):832–8.
58. Holden EC, et al. Improved outcomes after blastocyst-stage fro-zен-thawed embryo transfers compared with cleavage stage: A society for assisted reproductive technologies clinical outcomes reporting system study. *Fertil Steril.* 2018;110(1):89–94.e2.
59. Surrey E, et al. Freeze-all: Enhanced outcomes with cryopreser-vation at the blastocyst stage versus pronuclear stage using slow-freeze techniques. *Reprod Biomed Online.* 2010;21(3):411–7.
60. Youssry M, et al. Current aspects of blastocyst cryopreservation. *Reprod Biomed Online.* 2008;16(2):311–20.
61. Kader AA, et al. Factors affecting the outcome of human blastocyst vitrification. *Reprod Biol Endocrinol.* 2009;7:99.
62. Sunkara SK, et al. The influence of delayed blastocyst formation on the outcome of frozen-thawed blastocyst transfer: A systematic review and meta-analysis. *Hum Reprod.* 2010;25(8):1906–15.
63. Carroll J, Depypere H, Matthews CD. Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. *J Reprod Fertil.* 1990;90(2):547–53.
64. Safari S, et al. Cosmetic micromanipulation of vitrified-warmed cleavage stage embryos does not improve ART outcomes: An ultrastructural study of fragments. *Reprod Biol.* 2017;17(3):210–17.
65. Kumasako Y, et al. The efficacy of the transfer of twice frozen-thawed embryos with the vitrification method. *Fertil Steril.* 2009;91(2):383–6.
66. Hallamaa M, et al. Pregnancy potential and perinatal outcomes of embryos cryopreserved twice: A case-control study. *Reprod Biomed Online.* 2021;43(4):607–13.
67. Murakami M, et al. Perinatal outcome of twice-frozen-thawed embryo transfers: A clinical follow-up study. *Fertil Steril.* 2011;95(8):2648–50.
68. Winston RM, Hardy K. Are we ignoring potential dangers of in vitro fertilization and related treatments? *Nat Cell Biol.* 2002;4 Suppl:s14–8.
69. Pelkonen S, et al. Physical health of singleton children born after frozen embryo transfer using slow freezing: A 3-year follow-up study. *Hum Reprod.* 2015;30(10):2411–8.
70. Sha T, et al. Pregnancy-related complications and perinatal out-comes resulting from transfer of cryopreserved versus fresh embryos in vitro fertilization: A meta-analysis. *Fertil Steril.* 2018;109(2):330–42.e9.
71. Maheshwari A, et al. Is frozen embryo transfer better for moth-ers and babies? Can cumulative meta-analysis provide a definitive answer? *Hum Reprod Update.* 2018;24(1):35–58.
72. Wennerholm UB, et al. Children born after cryopreservation of embryos or oocytes: A systematic review of outcome data. *Hum Reprod.* 2009;24(9):2158–72.
73. Pinborg A, et al. Large baby syndrome in singletons born after frozen embryo transfer (FET): Is it due to maternal factors or the cryotechnique? *Hum Reprod.* 2014;29(3):618–27.
74. Maheshwari A, Raja EA, Bhattacharya S. Obstetric And perinatal outcomes after either fresh or thawed frozen embryo transfer: An Analysis of 112,432 singleton pregnancies recorded in the human fertilisation and embryology authority Anonymized dataset. *Fertil Steril.* 2016;106(7):1703–8.
75. Roelens C, et al. Artificially prepared vitrified-warmed embryo transfer cycles are associated with an increased risk of pre-eclampsia. *Reprod Biomed Online.* 2022;44(5):915–22.
76. Saito K, et al. Endometrial preparation methods for frozen-thawed embryo transfer are associated with altered risks of hypertensive disorders of pregnancy, placenta accreta, and gestational diabetes mellitus. *Hum Reprod.* 2019;34(8):1567–75.
77. von Versen-Höynck F, et al. Increased preeclampsia risk and reduced aortic compliance with in vitro fertilization cycles in the absence of a corpus luteum. *Hypertension.* 2019;73(3):640–9.
78. Conrad KP, Baker VL. Corpus luteal contribution to maternal preg-nancy physiology and outcomes in assisted reproductive technolo-gies. *Am J Physiol Regul Integr Comp Physiol.* 2013;304(2):R69–72.
79. Baksh S, et al. Natural vs. programmed cycles for frozen embryo transfer: Study protocol for an investigator-initiated, randomized, controlled, multicenter clinical trial. *Trials.* 2021;22(1):660.

Introduction

Standard *in vitro* fertilization (IVF) practices follow four stages: (i) ovarian stimulation and monitoring, (ii) transvaginal ultrasound-guided oocyte retrieval (TUGOR), (iii) fertilization and embryo culture in the laboratory, and (iv) transfer of the embryos to the uterus [1]. Although the IVF process is considered a minor surgical procedure, it has the potential to cause pain, fear, and anxiety in the patient [2].

Particularly, needle aspiration of follicles during TUGOR can cause significant pain, as well as anxiety in a patient population already at higher risk for psychological distress due to the treatment of infertility. Pain management and optimal anaesthetic intervention during this procedure can address and alleviate these complications, especially when repeated interventions can compound negative experiences and outcomes [3].

In addition, the patient population, though largely young and healthy, increasingly includes women with severe disease status—both to treat their infertility and to provide pre-implantation genetic diagnosis (PGD) for the selection against severe genetic diseases. Thus, the anaesthesiologist must have a clear understanding of the patient's psychological and physiological extra-genital status in order to provide optimal anaesthetic care [4].

Different anaesthetic techniques, including general anaesthesia, regional anaesthesia, and alternative medicine approaches, have all been used for these procedures. These techniques demand the active involvement of an anaesthesiologist to make transvaginal oocyte retrieval a safe and painless procedure for the benefit of the patient's emotional and physical well-being. The following sections outline the anaesthesiologist's role in caring for women during TUGOR.

Sedation

Sedation is a drug-induced depression of consciousness, a continuum culminating in general anaesthesia. The American Society of Anesthesiologists (ASA) defines three levels of sedation. *Minimal sedation*, also known as anxiolysis, or twilight sleep, is a drug-induced state during which the patient responds normally to verbal commands. Cognitive function and physical coordination may be impaired, but airway reflexes and ventilatory and cardiovascular functions are unaffected. *Moderate sedation* is a drug-induced state, also known as conscious sedation, where the patient responds purposefully to verbal commands, either alone or accompanied by light tactile stimulation. It should be noted that reflex withdrawal to painful stimuli is not considered a purposeful response. *Deep sedation* is a drug-induced state where the patient cannot easily be aroused but responds purposefully to repeated or painful stimulation [5]. It may be accompanied by clinically significant ventilatory depression. Increasing depth of sedation is accompanied by an escalation in the level of competency required to ensure safe sedation practice. The boundaries

between the different levels are not always clear and often there is a rapid transition between states of consciousness.

A major benefit of sedation is that it allows the procedure to be conveniently performed in an outpatient setting, and thus it is the most commonly used method of analgesia and anaesthesia during TUGOR [6, 7]. In comparison, 16% of UK clinics use general anaesthesia with tracheal intubation for IVF procedures, and 84% used intravenous sedation [8, 9].

A variety of methods of anaesthesia have been used for oocyte recovery. Drugs used for these procedures are selected by the quality of sedation and analgesia and their potentially deleterious effects on reproductive outcomes [10]. According to an updated Cochrane review conducted in 2018 [11], the various approaches for sedation used for IVF appeared acceptable and were associated with a high degree of women's satisfaction. However, evidence was insufficient to show conclusively whether any of the interventions influenced pregnancy rates. Sedation combined with analgesia such as opiates and further enhanced by paracervical blocks (PCB) or acupuncture techniques resulted in better pain relief than with one modality alone.

Surveys in the United States and the United Kingdom showed that sedation with a combination of opioids and benzodiazepines and/or propofol was the most frequently used combination of anaesthesia, with rates of 95% and 84%, respectively [6, 12]. In order to prevent the local burning sensation associated with propofol, pre-treatment with 1 mg/kg intravenous lidocaine may be helpful [13].

During sedation, anaesthesiologists should monitor the patient's vitals using the standard ASA monitoring standards, which include non-invasive blood pressure, heart rate, oxygen saturation, electrocardiogram, capnography, respiratory rate, and level of consciousness.

Pregnancy rate

One study reported live birth per woman [14]. Two studies reported ongoing pregnancy per woman [15, 16], and 13 additional studies reported clinical pregnancy rate per woman [14–26]. It is difficult to compare studies that consider different outcome measures: the number of collected and matured oocytes, embryo quality, fertilization, cleavage, implantation, abortion, pregnancy and/or delivery rates, or the study of plasma and follicular fluid concentrations of the anaesthetic agent, plasma and follicular levels of prolactin, progesterone, and cortisol.

Evidence was insufficient to show conclusively whether any of the interventions influenced pregnancy rates.

Pain control during TUGOR

Although many of the drugs used in anaesthesia have been found in the follicular fluid during TUGOR, adverse effects on oocytes have not been proven [27]. The pain experienced during aspiration of oocytes has been described as intense menstrual pain. The pain is produced by needle insertion through the vaginal wall and by mechanical stimulation of the ovary [19]. The number of

follcles retrieved and the duration of the procedure may affect pain intensity. Single-follicle aspiration is a shorter and less painful procedure than multiple-follicle aspiration. A favourable analgesic regimen for oocyte retrieval must have rapid onset, rapid recovery, with ease of administration and monitoring, while having no deleterious effects on the oocyte.

Kwan et al. [11] identified 24 randomized controlled trials involving 3160 women comparing the effects of five different methods of sedation and pain relief, including general anaesthesia with tracheal intubation. Although opioids are the most common drugs used for analgesia, there was insufficient evidence to support any method as superior to others in terms of pain relief or pregnancy outcomes. The analgesic effect of most methods was usually enhanced by the addition of another analgesic modality such as PCM, the injection of local anaesthetic into the cervix prior to egg retrieval.

In a randomized trial, Ng et al. [23] found that patients who received paracervical block plus placebo during egg collection experienced two and a half times higher levels of vaginal and abdominal pain than those who received both PCB and sedation.

In a randomized double-blinded non-inferiority trial of 170 infertile women undergoing TUGOR under sedation, Lai et al. [28] compared fentanyl and midazolam versus pethidine and diazepam for pain relief under sedation. The fentanyl and midazolam group had significantly lower vaginal and abdominal pain levels during oocyte retrieval than the pethidine and diazepam group.

Edwards et al. [29] reported on 4342 patients in the United Kingdom who were administered propofol (target-controlled infusion) and alfentanil boluses by non-anaesthetists during oocyte retrieval. According to the study design, safety was acceptable, with a respiratory adverse incident rate of 0.5/1000. In this study, unplanned, direct anaesthetic assistance was required in 3.5/1000 cases, and anaesthetic advice was required in 7.5% of cases.

We can conclude that no single sedation delivery system method appeared superior in terms of pain relief, pregnancy rates, and patient satisfaction. Future studies need to be consistent in the methods used to measure pain and the timing of such evaluations.

Complications of sedation

A Cochrane review in 2018 reported no serious adverse effects or oocyte retrieval procedure cancellations attributed to sedation [11]. Loss of airway control was very rare [26]. Coskun et al. [18] compared different doses of target-controlled MAC (monitored anaesthesia care) infusion (remifentanil 1.5 ng/mL, 2 ng/mL, and 2.5 ng/mL respectively). They reported that five women needed a jaw thrust followed by brief periods of assisted masked ventilation. Another study compared patient-controlled MAC with propofol versus patient-controlled MAC with midazolam and found that one participant in the midazolam group became transiently unresponsive and two women in the propofol group reported syncope [30].

Postoperative nausea and vomiting are a common problem after TUGOR and is related to peak plasma level of oestradiol and any previous history of postoperative nausea and vomiting [31]. Sedation plus PCB was associated with a lower likelihood of nausea and vomiting when compared with sedation only [32].

Drugs used in sedation

Midazolam (a benzodiazepine) is a commonly used drug in sedation because of its sedative and anxiolytic effects [28].

Additionally, it has anticonvulsant and amnesic (produces anterograde amnesia) effects. Opioids, mainly fentanyl, have a synergistic effect with midazolam and may enhance sedation, perhaps leading to respiratory depression or even apnoea; therefore, reduced doses of both drugs are mandatory. A minimal concentration of midazolam found in the follicular fluid has no detrimental effects on fertilization in animal or human studies [33–35]. Adverse effects associated with midazolam (such as drowsiness, confusion, or respiratory depression) can be reversed with Anexate (flumazenil).

Opioids (meperidine, fentanyl, alfentanil, and remifentanil) are narcotic agents widely used for general anaesthesia and MAC, mainly for their potent analgesic effects. Opioids may cause respiratory depression, bradycardia, and muscle rigidity in high doses or rapid administration. Apnoea or chest stiffness may necessitate manual ventilation or administration of naloxone or a muscle relaxant, followed by tracheal intubation. Other adverse effects of opioids are nausea, vomiting, and pruritus. Most adverse effects can be reversed by naloxone administration. The duration of effect of naloxone is relatively short and there is often re-narcotization, the recurrence of opioid side effects.

Remifentanil is a potent synthetic, ultra-short-acting opioid with a fast onset and short elimination time [36]. Remifentanil can be used for pain relief in the form of patient-controlled analgesia [15].

Propofol is the most popular anaesthetic induction agent, with a fast onset and short elimination time [29]. Propofol is used both for monitored anaesthesia care and general anaesthesia. Its administration is associated with decreased postoperative nausea and vomiting, primarily when used alone [37]. When administered through a peripheral vein, it causes a local burning pain sensation. The use of propofol in monitored anaesthesia care for oocyte retrieval necessitates anaesthesiologist or personnel skilled in airway management [29]. Depending on the dose, it may cause respiratory and myocardial depression. The use of propofol in combination with fentanyl or alfentanil in such a setting was found to be beneficial [12]. Goutziomitrou et al. [38] compared clinical outcomes of IVF cycles using propofol or thiopental sodium as anaesthetic agents for oocyte retrieval. The study revealed that the use of propofol compared with sodium thiopental for general anaesthesia during oocyte retrieval resulted in similar fertilization rates and IVF outcomes. Propofol-based techniques are well tolerated, even though lengthy procedures might lead to administering high propofol doses that would accumulate in follicular fluid [39].

The use of entropy monitoring to guide hypnotic administration versus conventional monitoring permitted a significant reduction of intraoperative propofol consumption, and its ability to differentiate between hypnotic and analgesic components of general anaesthesia reduced the need for postoperative analgesia [40].

In the past, egg allergy was a contraindication to propofol administration. However, according to the recent medical literature, propofol is likely to be safe in most of the egg-allergic patients who do not have a history of egg anaphylaxis [41, 42].

Ketamine is an old induction agent used in general anaesthesia and as a sedative and analgesic agent in monitored anaesthesia care. It belongs to the phencyclidine family of drugs that via its central nervous system (CNS) effect causes dissociative anaesthesia—a cataleptic condition with open eyes and a slow nystagmus gaze). It was considered an ideal anaesthetic agent due to several beneficial properties required for general anaesthesia, including

analgesia, loss of consciousness, and anterograde amnesia and preserved laryngeal reflexes while avoiding cardiorespiratory system-depressant effects seen with the use of other sedative hypnotics such as propofol. However, ketamine popularity has declined because of its postoperative psychological adverse effects in 5%–30% of patients, such as hallucinations, vivid dreaming, and feelings of excitement or fear that may last for several hours [17]. Ketamine's psychoactive deleterious effects can be reduced with the pre-emptive use of midazolam, thus rendering a ketamine–midazolam regimen as an excellent alternative to general anaesthesia with propofol and midazolam [17].

Ketamine administration may increase prolactin and β -endorphin levels. One study concluded that ketamine use during TUGOR can affect fertility rate compared to propofol [43]. Long durations of anaesthesia also seem to decrease implantation and clinical pregnancy rates.

Neuraxial anaesthesia

Neuraxial anaesthesia is an effective method of analgesia for TUGOR. It can be achieved by injection of local anaesthetics into the epidural or spinal space. Neuraxial anaesthesia has the advantage of minimal systemic local anaesthetic absorption, and therefore minimal follicular accumulation.

Two studies demonstrated that spinal anaesthesia increases the chance of fertilization success compared to general anaesthesia [44, 45]. Another study comparing MAC to spinal anaesthesia found that pregnancy rates were not significantly different between the spinal anaesthesia and MAC groups; however, the procedure duration was shorter in the spinal anaesthesia group than in the MAC group [46].

Martin et al. [47] demonstrated that patient comfort was improved when fentanyl was added to lidocaine during spinal anaesthesia for egg retrieval procedures when compared to lidocaine alone. In addition, postoperative narcotic requirements in the post-anaesthesia care unit (PACU) were reduced.

Adverse effects of epidural anaesthesia are post-dural puncture headache, urinary retention, and accidental intravascular injection of local anaesthetics. A high spinal block can cause respiratory depression. Local infection at the injection site, coagulopathy, increased intracranial pressure, and patient refusal are contraindications to epidural or spinal anaesthesia.

General anaesthesia

In the early days, laparoscopy under general anaesthesia was the predominant method of oocyte retrieval for human IVF procedures [48]. General anaesthesia was indicated for gamete intrafallopian transfer (GIFT) and zygote intrafallopian transfer (ZIFT) procedures [49].

The ideal regimen of general anaesthesia would reduce pain without the risk of adverse events, thus improving the positive experience for the patient, and thus mitigating future procedure-associated stress [17].

Intravenous hypnotics such as propofol or, less commonly, thiopental are used for induction agents for general anaesthesia. Subsequently, short- or intermediate-acting muscle relaxants that optimize intubation and surgical laparoscopic conditions with opiates such as fentanyl or alfentanil are administered. Maintenance of anaesthesia is maintained by a mixture of oxygen and air with nitrous oxide (N_2O) or inhaled volatile anaesthetic agents such as isoflurane, sevoflurane, or desflurane. A

total intravenous anaesthesia (TIVA) technique can be an alternative to the use of volatile anaesthetics. After the procedure is completed and when indicated, patients usually receive a reversal agent to avoid residual relaxation prior to extubation.

General anaesthesia has side effects and complications such as drowsiness, postoperative nausea and vomiting, throat pain, and muscle pain, in addition to airway complications such as airway trauma, dental injury, and difficulty in ventilation or intubation. Other serious complications can arise, such as aspiration, severe allergic reaction, and malignant hyperthermia. The main concern related to IVF procedures is that the medications used during general anaesthesia may negatively affect fertilization and cleavage rate.

Studies have suggested that nitrous oxide and volatile anaesthetic agents interfere with some aspects of reproductive physiology *in vitro*. The use of nitrous oxide for sedation or general anaesthesia is controversial. By inhibiting methionine synthase, nitrous oxide decreases thymidine production, which is needed for DNA production [50]. Studies show that nitrous oxide impairs the function of mitotic spindles in cell cultures and leads to lower pregnancy and delivery rates [51, 52]. Other studies comparing fertilization and cleavage rates of mature oocytes following general anaesthesia with nitrous oxide versus intravenous sedation showed lower fertilization rate with prolonged general anaesthesia [53]. Warren et al. [54] reported that brief exposure of mouse pre-implantation embryos to nitrous oxide may be deleterious to subsequent embryo cleavage. However, Rosen et al. [55], who studied the effect of nitrous oxide on IVF success rates in women undergoing laparoscopic oocyte retrieval under isoflurane-based general anaesthesia, failed to demonstrate an adverse effect. They concluded that use of nitrous oxide may actually increase the success rates of IVF by reducing the concentrations of other potentially toxic and less diffusible anaesthetics.

Data suggest that volatile halogenated anaesthetic agents can also affect embryo development *in vitro*. Chetkowski et al. [56] reported that isoflurane significantly inhibited mouse embryo development *in vitro*. Eger et al. [57] reported that Compound A, a degradation product of sevoflurane, increases sister chromatid exchanges and has been associated with genotoxic ovarian cell effects. Pirol et al. [31] reported that anaesthesia with sevoflurane had a lower percentage of viable embryos.

General anaesthesia may increase prolactin levels and stress hormones such as cortisol and epinephrine, which might have adverse embryonic effects [58, 59]. In 1987, Hayes et al. [60] found that prolonged exposure to general anaesthesia and CO_2 pneumoperitoneum adversely affects oocyte cleavage rate and maturity. Similarly, Boyers et al. reported that laparoscopic aspiration of oocytes under general anaesthesia (i.e. isoflurane or enflurane with a 50% nitrous–oxygen mixture) and CO_2 pneumoperitoneum adversely affect oocyte quality [48]. They recommend minimizing the exposure time to both general anaesthesia and CO_2 pneumoperitoneum.

General anaesthesia has also been compared with MAC. Wilhelm et al. [61] found lower pregnancy rates in women undergoing TUGOR under general anaesthesia (alfentanil, propofol, isoflurane in combination with 60% nitrous oxide in oxygen) versus monitored anaesthesia care with remifentanil.

The accumulation of propofol in follicular fluid during general anaesthesia has been demonstrated by Coetsier et al. [62]. The author recommends keeping the retrieval procedure as short as possible in order to limit anaesthetic drug exposure. Palot et al. [63] showed a lower cleavage rate following oocyte retrieval when nitrous oxide and propofol in continuous infusion were used.

Vincent et al. [64] showed that propofol–nitrous oxide anaesthesia was associated with lower clinical and ongoing pregnancy rates compared with isoflurane–nitrous oxide anaesthesia for laparoscopic pronuclear stage transfer (PROST).

Despite the concerns regarding general anaesthesia use, in a study by Christiaens et al. [65] general anaesthesia with propofol and a 50% oxygen–air mixture was found to be associated with fertilization, cleavage, and pregnancy rates similar to those produced by a PCB with local anaesthetic. In a study reported by Beilin et al. the induction and maintenance of general anaesthesia with propofol, nitrous oxide, isoflurane, or midazolam for GIFT procedures demonstrated no agent-related differences in pregnancy rates [49]. A Cochrane review concluded that there is no particular pain relief method that appears to be more effective for IVF; nor is there significant differences regarding pregnancy rates or patient satisfaction [11].

Although volatile agents continue to be used for reproductive procedures, caution should be taken, and further studies are needed to investigate their effect on IVF outcomes [26].

Paracervical and pre-ovarian block

PCB and pre-ovarian block (POB) techniques are used in order to modulate pain during oocyte retrieval in IVF and may be used alone or in combination with conscious sedation [66–68]. PCB is also used for labour analgesia, uterine curettage, and hysteroscopy [69, 70].

In PCB, the local anaesthetic is usually injected in four locations around the cervix in the vaginal mucosa. In the POB technique a local anaesthetic, usually lidocaine, is injected between the vaginal wall and the peritoneal surface near the ovary [67].

In a prospective controlled trial, Cerne et al. [67] conclude that no differences were found in the overall pain experienced during the oocyte retrieval procedure with POB compared to PCB.

PCB with lidocaine is recommended for use in combination with sedation to reduce the pain of the procedure [71].

Lidocaine is the most common local anaesthetic used in PCB. There seems to be no consensus regarding the most effective lidocaine dose in studies that reported the use of 50, 100, 150 and 200 mg [66, 68, 71, 72]. No differences were found in pain levels during oocyte retrieval with different doses of lidocaine, thus, the lowest dose is recommended [66].

A possible risk associated with nerve blocks is the potential for local anaesthetic toxicity [72, 73].

Wikland et al. [72] studied the concentration of lidocaine in follicular fluid and showed that there were no adverse effects on fertilization, cleavage, or pregnancy rates when using PCB. Ng et al. [66] confirmed that IVF outcome was not affected, even by larger doses of lidocaine such as 200 mg.

Randomized controlled trials compared the effects of PCB combined with conventional analgesia to PCB with electroacupuncture. Intraoperative analgesic scores were lower in the group that received conventional analgesics with PCB, but there were no significant differences regarding clinical pregnancy rates [14, 16, 21].

Alternative and non-pharmacological pain management

Acupuncture is one of the most commonly used alternative medical procedures worldwide [10]. Acupuncture has a prominent place in traditional Chinese medicine. It has gained popularity in

Western countries as a treatment modality of chronic pain, fibromyalgia, drug addiction, and as an adjunct in fertility treatment and pregnancy [74, 75]. There are no comprehensive explanations of the mechanism of action. Acupuncture has been shown to increase the b-endorphin levels with anxiolytic properties and cortical activity [74, 76, 77]. Acupuncture can reduce high impedance in the uterine arteries by sympathetic inhibition via the endorphin system and by central sympatholytic activity, which increase uterine blood flow and myometrial activity [78, 79].

The role of acupuncture in assisted reproductive techniques is under investigation. Several systematic reviews and meta-analyses analysed the effects of acupuncture among women undergoing IVF, with varying results. The meta-analysis published by Manheimer et al. [80] indicated that acupuncture at the time of embryo transfer improved clinical pregnancy rates. In two other meta-analyses, no beneficial effects of acupuncture had been shown on clinical pregnancy rates or live birth rates. The authors of these meta-analyses indicated bias effects as a result of the heterogeneity of the trials, use of sham acupuncture (use of non-selected points) as control or lack of control, and the heterogeneity of procedures [81, 82]. In the meta-analysis published in 2015 by Matsota et al. [10], of a total of 16 trials that met the selection criteria, 8 studies confirm the beneficial effects of acupuncture on IVF, while 6 studies failed to find significant differences. Two studies concluded that the use of acupuncture reduces IVF outcome. Another meta-analysis published in 2019 by Xie et al. [83] that included 27 studies, found acupuncture beneficial for IVF outcomes in women with a history of unsuccessful IVF attempts. In this analysis, the number of acupuncture treatments was found to influence outcomes.

Acupuncture may be an alternative for women desiring a non-pharmacological method [19]. In a recent multicentre randomized clinical trial with a total of 848 women undergoing IVF, Smith et al. [84] conclude that administration of acupuncture versus sham acupuncture around the time of ovarian stimulation and embryo transfer did not result in statistically significantly different live birth rates. These findings do not support the use of acupuncture to improve the rate of live births among women undergoing IVF. More research is needed with a greater number of subjects to elucidate the role of acupuncture on IVF outcome.

Anaesthesia for pre-implantation genetic testing (PGT)

In recent years, a notable percentage of women undergoing IVF treatment do so in order to perform pre-implantation genetic testing (PGT), formerly known as pre-implantation genetic diagnosis (PGD). In most cases, pairs approaching a PGD test do not do so due to infertility, but are rather individuals who have a family history of genetic disorders or who suffer from a genetic disease themselves. Thus, the reason for undergoing this procedure is to prevent the transfer of a genetic disorder to their offspring [85].

The anaesthetic management of such patients may significantly differ from that of a regular IVF treatment. Diseases such as Marfan syndrome, Huntington's disease, myotonic dystrophy, and cystic fibrosis may present a professional challenge to anaesthesiologists, especially in cases of an active disease with significant symptoms [86, 87]. Most autosomal dominant disorders lead to phenotypic expressions of the disease, and demand previous knowledge and preparation of the anaesthesiologist.

One of the options for an early inspection by the anaesthesiologist is a summoning of each patient who will undergo PGT to an outpatient anaesthetic clinic. Nevertheless, patients with no clinical expression of the disorder, as in cases of X-linked Duchenne muscular dystrophy, also demand some pre-procedural preparation by the anaesthesiologist. The inspection at the anaesthesia clinic is also important for the evaluation of the patient's functional level, collection of an anaesthesiologic anamnesis, and evaluation of the airways [88].

Patients suffering of conditions such as myotonic dystrophy or Huntington's disease may approach PGT when already in a state of muscular weakness, and thus the performance of general anaesthesia or sedation in these cases might lead to a necessity of mechanical ventilation. The anaesthetic alternative for such patients may be regional anaesthesia, similarly to patients with cystic fibrosis in advanced stages [89].

Another reason for the importance of an early inspection of these patients by an anaesthesiologist is the consideration of the need for additional examinations by a pulmonologist or cardiologist, or the performance of additional tests such as a pulmonary function test, echocardiography, and Holter monitoring. For example, patients with myotonic dystrophy may suffer from atrioventricular arrhythmias, heart failure, and a reduced ejection fraction [90]; thus, echocardiography, optimal stabilization of arrhythmias, and optimization of the general condition are crucial as part of the pre-procedural anaesthetic management in such cases. Airways evaluation and an early diagnosis of potentially difficult airways in symptomatic patients may also be a reason to avoid general anaesthesia and recommend regional anaesthesia instead.

In large medical centres treating difficult patients approaching PGT procedures, the maintenance of clear guidelines for the treatment in cases with specific diagnoses is recommended. For example, patients with myotonic dystrophy should approach an anaesthesiologic examination with pre-prepared, up-to-date, echocardiography and ECG results—allowing the anaesthesiologist to accurately analyse the patient's condition and build a maximal-safety anaesthetic approach [4].

It is noteworthy that in some cases, patients already approach PGT procedures with a severe underlying condition not enabling pregnancy or childbirth—in aim to undergo surrogacy procedures. In these cases, it is crucial that IVF for PGT is performed in a multidisciplinary centre with full anaesthesiologic, ICU, and recovery services as backup [91].

Conclusions

The role of the anaesthesiologist in IVF is to provide adequate comfort and pain relief to patients and to allow optimal working conditions for gynaecologists during oocyte retrieval procedures. How to provide appropriate anaesthetic assistance during the procedure depends on the patient's cooperation and general condition.

If the patient is comfortable and cooperative, conscious sedation is a good option. However, in some cases, regional or general anaesthesia may be required. Currently, there is not enough evidence to definitively show whether any one of the anaesthetic choices affects pregnancy rates. There is also no data to support that there is a single method of anaesthesia that is preferable in terms of pain relief and patient satisfaction.

The duration of anaesthesia should be as short as possible with minimal risk of post-anaesthesia complications. Extra precaution should be taken when using new and less-investigated medications, as their effect on IVF outcomes is not fully known.

Concomitant or related diseases and therapy with some medications may influence the choice of anaesthesia for IVF. Due to the increasing utilization of IVF procedures, not only due to infertility but also for PGT and due to more common work with patients suffering from different extragenital pathologies, it is necessary to plan in advance the entire anaesthetic process, including a preliminary meeting with the anaesthetist and performance of additional tests and preparations when necessary. All of the preceding greatly emphasizes the importance of collaboration and good cooperation between the gynaecologist and the anaesthesiologist.

References

- Gindoff PR, Hall JL, Stillman RJ. Utility of in vitro fertilization at diagnostic laparoscopy. *Fertil Steril*. 1994;62(2):237–41. doi: [10.1016/s0015-0282\(16\)56871-x](https://doi.org/10.1016/s0015-0282(16)56871-x).
- Tang J, Gibson SJ. A psychophysical evaluation of the relationship between trait anxiety, pain perception, and induced state anxiety. *J Pain*. 2005;6(9):612–9. doi: [10.1016/j.jpain.2005.03.009](https://doi.org/10.1016/j.jpain.2005.03.009).
- Vlahos NF, Giannakou I, Vlachos A, Vitoratos N. Analgesia and anesthesia for assisted reproductive technologies. *Int J Gynaecol Obstet*. 2009;105(3):201–5. doi: [10.1016/j.ijgo.2009.01.017](https://doi.org/10.1016/j.ijgo.2009.01.017).
- Ioscovich A, Eldar-Geva T, Weitman M, Altarescu G, Rivilis A, Elstein D. Anesthetic management for oocyte retrieval: An exploratory analysis comparing outcome in in vitro fertilization cycles with and without pre-implantation genetic diagnosis. *J Hum Reprod Sci*. 2013;6(4):263–6. doi: [10.4103/0974-1208.126303](https://doi.org/10.4103/0974-1208.126303).
- Das S, Ghosh S. Monitored anesthesia care: An overview. *J Anaesthesiol Clin Pharmacol*. 2015;31(1):27–9. doi: [10.4103/0970-9185.150525](https://doi.org/10.4103/0970-9185.150525).
- Ditkoff EC, Plumb J, Selick A, Sauer MV. Anesthesia practices in the United States common to in vitro fertilization (IVF) centers. *J Assist Reprod Genet*. 1997;14(3):145–7. doi: [10.1007/BF02766130](https://doi.org/10.1007/BF02766130).
- Trout SW, Vallerand AH, Kemmann E. Conscious sedation for in vitro fertilization. *Fertil Steril*. 1998;69(5):799–808. doi: [10.1016/s0015-0282\(98\)00031-4](https://doi.org/10.1016/s0015-0282(98)00031-4).
- Elkington NM, Kehoe J, Acharya U. Intravenous sedation in assisted conception units: A UK survey. *Hum Fertil (Camb)*. 2003;6(2):74–6. doi: [10.1080/1464770312331369083](https://doi.org/10.1080/1464770312331369083).
- Andersen AN, Goossens V, Ferrari AP, et al. Assisted reproductive technology in Europe, 2004: Results generated from European registers by ESHRE. *Hum Reprod*. 2008;23:756–71.
- Matsots P, Kaminioti E, Kostopanagiotou G. Anesthesia related toxic effects on in vitro fertilization outcome: Burden of proof. *Biomed Res Int*. 2015;2015:475362. doi: [10.1155/2015/475362](https://doi.org/10.1155/2015/475362).
- Kwan I, Wang R, Pearce E, Bhattacharya S. Pain relief for women undergoing oocyte retrieval for assisted reproduction. *Cochrane Database Syst Rev*. 2018;5(5):CD004829. doi: [10.1002/14651858.CD004829.pub4](https://doi.org/10.1002/14651858.CD004829.pub4).
- Elkington NM, Kehoe J, Acharya U, Policy and Practice Committee of the British Fertility Society. Recommendations for good practice for sedation in assisted conception. *Hum Fertil (Camb)*. 2003;6(2):77–80. doi: [10.1080/1464770312331369093](https://doi.org/10.1080/1464770312331369093).
- Euasobhon P, Dej-Arkam S, Siriussawakul A, et al. Lidocaine for reducing propofol-induced pain on induction of anaesthesia in adults. *Cochrane Database Syst Rev*. 2016;2(2):CD007874. doi: [10.1002/14651858.CD007874.pub2](https://doi.org/10.1002/14651858.CD007874.pub2).
- Stener-Victorin E, Waldenström U, Nilsson L, Wiklund M, Janson PO. A prospective randomized study of electro-acupuncture versus alfentanil as anaesthesia during oocyte aspiration in in-vitro fertilization. *Hum Reprod*. 1999;14(10):2480–4. doi: [10.1093/humrep/14.10.2480](https://doi.org/10.1093/humrep/14.10.2480).
- Lier MC, Douwenga WM, Yilmaz F, et al. Patient-controlled remifentanil analgesia as alternative for pethidine with midazolam during oocyte retrieval in IVF/ICSI procedures: A randomized controlled trial. *Pain Pract*. 2015;15(5):487–95. doi: [10.1111/papr.12189](https://doi.org/10.1111/papr.12189).

16. Stener-Victorin E, Waldenström U, Wikland M, Nilsson L, Hägglund L, Lundeberg T. Electro-acupuncture as a peroperative analgesic method and its effects on implantation rate and neuropeptide Y concentrations in follicular fluid. *Hum Reprod.* 2003;18(7):1454–60. doi: [10.1093/humrep/deg277](https://doi.org/10.1093/humrep/deg277).
17. Ben-Shlomo I, Moskovich R, Katz Y, Shalev E. Midazolam/ketamine sedative combination compared with fentanyl/propofol/isoflurane anaesthesia for oocyte retrieval. *Hum Reprod.* 1999;14(7):1757–9. doi: [10.1093/humrep/14.7.1757](https://doi.org/10.1093/humrep/14.7.1757).
18. Coskun D, Gunaydin B, Tas A, Inan G, Celebi H, Kaya K. A comparison of three different target-controlled remifentanil infusion rates during target-controlled propofol infusion for oocyte retrieval. *Clinics (Sao Paulo).* 2011;66(5):811–5. doi: [10.1590/s1807-59322011000500017](https://doi.org/10.1590/s1807-59322011000500017).
19. Gejervall AL, Stener-Victorin E, Möller A, Janson PO, Werner C, Bergh C. Electro-acupuncture versus conventional analgesia: A comparison of pain levels during oocyte aspiration and patients' experiences of well-being after surgery. *Hum Reprod.* 2005;20(3):728–35. doi: [10.1093/humrep/deh665](https://doi.org/10.1093/humrep/deh665).
20. Guasch E, Ardyo M, Cuadrado C, González Gancedo P, González A, Gilsanz F. Comparación de cuatro técnicas anestésicas para fecundación in vitro [comparison of 4 anesthetic techniques for in vitro fertilization]. *Rev Esp Anestesiol Reanim.* 2005;52(1): 9–18.
21. Humaidan P, Stener-Victorin E. Pain relief during oocyte retrieval with a new short duration electro-acupuncture technique—an alternative to conventional analgesic methods. *Hum Reprod.* 2004;19(6):1367–72. doi: [10.1093/humrep/deh229](https://doi.org/10.1093/humrep/deh229).
22. Matsota P, Sidiropoulou T, Batistaki C, et al. Analgesia with remifentanil versus anesthesia with propofol-alfentanil for transvaginal oocyte retrieval: A randomized trial on their impact on in vitro fertilization outcome. *Middle East J Anaesthesiol.* 2012;21(5): 685–92.
23. Ng EH, Chui DK, Tang OS, Ho PC. Paracervical block with and without conscious sedation: A comparison of the pain levels during egg collection and the postoperative side effects. *Fertil Steril.* 2001;75(4):711–7. doi: [10.1016/s0015-0282\(01\)01693-4](https://doi.org/10.1016/s0015-0282(01)01693-4).
24. Ozturk E, Gunaydin B, Karabacak O, Tuncer B, Erdem M, Erdem A, et al. Remifentanil infusion and paracervical block combination versus remifentanil infusion alone during in vitro fertilisation (IVF). *Turkish J Med Sci.* 2006;36:105–11.
25. Sator-Katzenschlager SM, Wölfler MM, Kozek-Langenecker SA, et al. Auricular electro-acupuncture as an additional perioperative analgesic method during oocyte aspiration in IVF treatment. *Hum Reprod.* 2006;21(8):2114–20. doi: [10.1093/humrep/del110](https://doi.org/10.1093/humrep/del110).
26. Thompson N, Murray S, MacLennan F, et al. A randomised controlled trial of intravenous versus inhalational analgesia during outpatient oocyte recovery. *Anesthesia.* 2000;55(8):770–3. doi: [10.1046/j.1365-2044.2000.01468.x](https://doi.org/10.1046/j.1365-2044.2000.01468.x).
27. Sharma A, Borle A, Trikha A. Anesthesia for in vitro fertilization. *J Obstet Anaesth Crit Care.* 2015;5:62–72.
28. Lai SF, Lam MT, Li HWR, Ng EHY. A randomized double-blinded non-inferiority trial comparing fentanyl and midazolam with pethidine and diazepam for pain relief during oocyte retrieval. *Reprod Biomed Online.* 2020;40(5):653–60. doi: [10.1016/j.rbmo.2020.01.021](https://doi.org/10.1016/j.rbmo.2020.01.021).
29. Edwards JA, Kinsella J, Shaw A, Evans S, Anderson KJ. Sedation for oocyte retrieval using target controlled infusion of propofol and incremental alfentanil delivered by non-anaesthetists. *Anesthesia.* 2010;65(5):453–61. doi: [10.1111/j.1365-2044.2010.06264.x](https://doi.org/10.1111/j.1365-2044.2010.06264.x).
30. Cook LB, Lockwood GG, Moore CM, Whitwam JG. True patient-controlled sedation. *Anesthesia.* 1993;48(12):1039–44. doi: [10.1111/j.1365-2044.1993.tb07521.x](https://doi.org/10.1111/j.1365-2044.1993.tb07521.x).
31. Piroli A, Marci R, Marinangeli F, et al. Comparison of different anaesthetic methodologies for sedation during in vitro fertilization procedures: Effects on patient physiology and oocyte competence. *Gynecol Endocrinol.* 2012;28(10):796–9. doi: [10.3109/09513590.2012.664193](https://doi.org/10.3109/09513590.2012.664193).
32. Gunaydin B, Ozulgen IK, Ozturk E, Tekgul ZT, Kaya K. Remifentanil versus remifentanil with paracervical block on plasma remifentanil concentrations and pulmonary function tests for transvaginal ultrasound-guided oocyte retrieval. *J Opioid Manag.* 2007;3(5):267–72. doi: [10.5055/jom.2007.0014](https://doi.org/10.5055/jom.2007.0014).
33. Swanson RJ, Leavitt MG. Fertilization and mouse embryo development in the presence of midazolam. *Anesth Analg.* 1992;75(4):549–54. doi: [10.1213/00000539-199210000-00014](https://doi.org/10.1213/00000539-199210000-00014).
34. Chopineau J, Bazin JE, Terrisse MP, et al. Assay for midazolam in liquor folliculi during in vitro fertilization under anesthesia. *Clin Pharm.* 1993;12(10):770–3.
35. Jennings JC, Moreland K, Peterson CM. In vitro fertilisation. A review of drug therapy and clinical management. *Drugs.* 1996;52(3):313–43. doi: [10.2165/00003495-199652030-00002](https://doi.org/10.2165/00003495-199652030-00002).
36. Arndt M, Kreinemeyer J, Vagts DA, Nöldge-Schomburg GF. Remifentanil-Analgezie zur Follikelpunktion für In-Vitro-Fertilisation [Remifentanil analgesia for aspiration of follicles for oocyte retrieval]. *Anaesthesiol Reanim.* 2004;29(3):69–73.
37. Weibel S, Rücker G, Eberhart LH, et al. Drugs for preventing post-operative nausea and vomiting in adults after general anaesthesia: A network meta-analysis. *Cochrane Database Syst Rev.* 2020 Oct 19;10(CD012859). doi: [10.1002/14651858.CD012859.pub2](https://doi.org/10.1002/14651858.CD012859.pub2).
38. Goutziomitrou E, Venetis CA, Kolibianakis EM, et al. Propofol versus thiopental sodium as anaesthetic agents for oocyte retrieval: A randomized controlled trial. *Reprod Biomed Online.* 2015;31(6):752–9. doi: [10.1016/j.rbmo.2015.08.013](https://doi.org/10.1016/j.rbmo.2015.08.013).
39. Guasch E, Gómez R, Brogly N, Gilsanz F. Anesthesia and analgesia for transvaginal oocyte retrieval. Should we recommend or avoid any anesthetic drug or technique? *Curr Opin Anaesthesiol.* 2019;32(3):285–90. doi: [10.1097/ACO.0000000000000715](https://doi.org/10.1097/ACO.0000000000000715).
40. Tewari S, Bhadaria P, Wadhawan S, Prasad S, Kohli A. Entropy vs standard clinical monitoring using total intravenous anaesthesia during transvaginal oocyte retrieval in patients for in vitro fertilization. *J Clin Anesth.* 2016;34:105–12. doi: [10.1016/j.jclinane.2016.02.029](https://doi.org/10.1016/j.jclinane.2016.02.029).
41. Murphy A, Campbell DE, Baines D, Mehr S. Allergic reactions to propofol in egg-allergic children. *Anesth Analg.* 2011;113(1):140–4. doi: [10.1213/ANE.0b013e31821b450f](https://doi.org/10.1213/ANE.0b013e31821b450f).
42. Bradley AE, Tober KE, Brown RE. Use of propofol in patients with food allergies. *Anesthesia.* 2008;63(4):439. doi: [10.1111/j.1365-2044.2008.05505.x](https://doi.org/10.1111/j.1365-2044.2008.05505.x).
43. Tola EN. The effect of anesthetic agents for oocyte pick-up on in vitro fertilization outcome: A retrospective study in a tertiary center. *Taiwan J Obstet Gynecol.* 2019;58(5):673–9. doi: [10.1016/j.tjog.2019.07.016](https://doi.org/10.1016/j.tjog.2019.07.016).
44. Azmude A, Agha'amou S, Yousefshahi F, et al. Pregnancy outcome using general anesthesia versus spinal anesthesia for in vitro fertilization. *Anesth Pain Med.* 2013;3(2):239–42. doi: [10.5812/aapm.11223](https://doi.org/10.5812/aapm.11223)
45. Agha'amou S, Azmudeh A, Yousefshahi F, et al. Does spinal analgesia have advantage over general anesthesia for achieving success in in-vitro fertilization? *Oman Med J.* 2014;29(2):97–101. doi: [10.5001/omj.2014.24](https://doi.org/10.5001/omj.2014.24).
46. Heo HJ, Kim YY, Lee JH, Lee HG, Baek SM, Kim KM. Comparison of chemical pregnancy rates according to the anesthetic method during ultrasound-guided transvaginal oocyte retrieval for in vitro fertilization: A retrospective study. *Anesth Pain Med (Seoul).* 2020;15(1): 49–52. doi: [10.17085/apm.2020.15.1.49](https://doi.org/10.17085/apm.2020.15.1.49).
47. Martin R, Tsien LC, Tzeng G, Hornstein MD, Datta S. Anesthesia for in vitro fertilization: The addition of fentanyl to 1.5% lidocaine. *Anesth Analg.* 1999;88(3):523–6. doi: [10.1097/00000539-199903000-00010](https://doi.org/10.1097/00000539-199903000-00010).
48. Boyers SP, Lavy G, Russell JB, DeCherney AH. A paired analysis of in vitro fertilization and cleavage rates of first- versus last-recovered preovulatory human oocytes exposed to varying intervals of 100% CO₂ pneumoperitoneum and general anesthesia. *Fertil Steril.* 1987;48(6):969–74. doi: [10.1016/s0015-0282\(16\)59593-4](https://doi.org/10.1016/s0015-0282(16)59593-4).

49. Beilin Y, Bodian CA, Mukherjee T, et al. The use of propofol, nitrous oxide, or isoflurane does not affect the reproductive success rate following gamete intrafallopian transfer (GIFT): A multicenter pilot trial/survey. *Anesthesiology*. 1999;90(1):36–41. doi: [10.1097/00000542-199901000-00007](https://doi.org/10.1097/00000542-199901000-00007).
50. Hansen DK, Billings RE. Effects of nitrous oxide on macromolecular content and DNA synthesis in rat embryos. *J Pharmacol Exp Ther*. 1986;238(3):985–9.
51. Kieler J. The cytotoxic effect of nitrous oxide at different oxygen tensions. *Acta Pharmacol Toxicol (Copenh)*. 1957;13(3):301–8. doi: [10.1111/j.1600-0773.1957.tb00266.x](https://doi.org/10.1111/j.1600-0773.1957.tb00266.x).
52. Gonen O, Shulman A, Ghetler Y, et al. The impact of different types of anesthesia on in vitro fertilization-embryo transfer treatment outcome. *J Assist Reprod Genet*. 1995;12(10):678–82. doi: [10.1007/BF02212892](https://doi.org/10.1007/BF02212892).
53. Jensen JT, Boyers SP, Grunfeld LH, et al. Anesthesia exposure may affect fertilization rates in human oocytes collected by ultrasound aspiration. Presented at the Fifth World Congress on In Vitro Fertilization and Embryo Transfer. Norfolk, VA, 1987: 48.
54. Warren JR, Shaw B, Steinkampf MP. Effects of nitrous oxide on preimplantation mouse embryo cleavage and development. *Biol Reprod*. 1990;43(1):158–61. doi: [10.1095/biolreprod43.1.158](https://doi.org/10.1095/biolreprod43.1.158).
55. Rosen MA, Roizen MF, Eger EI 2nd, et al. The effect of nitrous oxide on in vitro fertilization success rate. *Anesthesiology*. 1987;67(1):42–4. doi: [10.1097/00000542-198707000-00007](https://doi.org/10.1097/00000542-198707000-00007).
56. Chetkowski RJ, Nass TE. Isofluorane inhibits early mouse embryo development in vitro. *Fertil Steril*. 1988;49(1):171–3.
57. Eger EI 2nd, Lester MJ, Winegar R, Han C, Gong D. Compound A induces sister chromatid exchanges in Chinese hamster ovary cells. *Anesthesiology*. 1997;86(4):918–22. doi: [10.1097/00000542-199704000-00022](https://doi.org/10.1097/00000542-199704000-00022).
58. Taylor PJ, Trounson A, Besanko M, Burger HG, Stockdale J. Plasma progesterone and prolactin changes in superovulated women before, during, and immediately after laparoscopy for in vitro fertilization and their relation to pregnancy. *Fertil Steril*. 1986;45(5):680–6. doi: [10.1016/s0015-0282\(16\)49341-6](https://doi.org/10.1016/s0015-0282(16)49341-6).
59. Lehtinen AM, Laatikainen T, Koskimies AI, Hovorka J. Modifying effects of epidural analgesia or general anesthesia on the stress hormone response to laparoscopy for in vitro fertilization. *J In Vitro Fert Embryo Transf*. 1987;4(1):23–29. doi: [10.1007/BF01555431](https://doi.org/10.1007/BF01555431).
60. Hayes MF, Sacco AG, Savoy-Moore RT, Magyar DM, Endler GC, Moghissi KS. Effect of general anesthesia on fertilization and cleavage of human oocytes in vitro. *Fertil Steril*. 1987;48(6):975–81. doi: [10.1016/s0015-0282\(16\)59594-6](https://doi.org/10.1016/s0015-0282(16)59594-6).
61. Wilhelm W, Hammadeh ME, White PF, Georg T, Fleser R, Biedler A. General anesthesia versus monitored anesthesia care with remifentanil for assisted reproductive technologies: Effect on pregnancy rate. *J Clin Anesth*. 2002;14(1):1–5. doi: [10.1016/s0952-8180\(01\)00331-2](https://doi.org/10.1016/s0952-8180(01)00331-2).
62. Coetsier T, Dhont M, De Sutter P, Merchiers E, Verschelen L, Rosseel MT. Propofol anaesthesia for ultrasound guided oocyte retrieval: Accumulation of the anaesthetic agent in follicular fluid. *Hum Reprod*. 1992;7(10):1422–4. doi: [10.1093/oxfordjournals.humrep.a137586](https://doi.org/10.1093/oxfordjournals.humrep.a137586).
63. Palot M, Harika C, Pigeon F, et al. Propofol in general anesthesia for I.V.F. (by vaginal and transurethral route) - follicular fluid concentration and cleavage rate. *Anesthesiology*. 1988;69:573.
64. Vincent RD Jr, Syrop CH, Van Voorhis BJ, et al. An evaluation of the effect of anesthetic technique on reproductive success after laparoscopic pronuclear stage transfer. Propofol/nitrous oxide versus isoflurane/nitrous oxide. *Anesthesiology*. 1995;82(2):352–8. doi: [10.1097/00000542-199502000-00005](https://doi.org/10.1097/00000542-199502000-00005).
65. Christiaens F, Janssenswillen C, Van Steirteghem AC, Devroey P, Verborgh C, Camu F. Comparison of assisted reproductive technology performance after oocyte retrieval under general anaesthesia (propofol) versus paracervical local anaesthetic block: A case-controlled study. *Hum Reprod*. 1998;13(9):2456–60. doi: [10.1093/humrep/13.9.2456](https://doi.org/10.1093/humrep/13.9.2456).
66. Ng EH, Tang OS, Chui DK, Ho PC. Comparison of two different doses of lignocaine used in paracervical block during oocyte collection in an IVF programme. *Hum Reprod*. 2000;15(10):2148–51. doi: [10.1093/humrep/15.10.2148](https://doi.org/10.1093/humrep/15.10.2148).
67. Cerne A, Bergh C, Borg K, et al. Pre-ovarian block versus paracervical block for oocyte retrieval. *Hum Reprod*. 2006;21(11):2916–21. doi: [10.1093/humrep/del271](https://doi.org/10.1093/humrep/del271).
68. Hammarberg K, Enk L, Nilsson L, Wikland M. Oocyte retrieval under the guidance of a vaginal transducer: Evaluation of patient acceptance. *Hum Reprod*. 1987;2(6):487–90. doi: [10.1093/oxfordjournals.humrep.a136575](https://doi.org/10.1093/oxfordjournals.humrep.a136575).
69. Wiebe ER. Comparison of the efficacy of different local anesthetics and techniques of local anesthesia in therapeutic abortions. *Am J Obstet Gynecol*. 1992;167(1):131–4. doi: [10.1016/s0002-9378\(11\)91645-7](https://doi.org/10.1016/s0002-9378(11)91645-7).
70. Vercellini P, Colombo A, Mauro F, Oldani S, Bramante T, Crosignani PG. Paracervical anesthesia for outpatient hysteroscopy. *Fertil Steril*. 1994;62(5):1083–5.
71. Ng EH, Tang OS, Chui DK, Ho PC. A prospective, randomized, double-blind and placebo-controlled study to assess the efficacy of paracervical block in the pain relief during egg collection in IVF. *Hum Reprod*. 1999;14(11):2783–7. doi: [10.1093/humrep/14.11.2783](https://doi.org/10.1093/humrep/14.11.2783).
72. Wikland M, Evers H, Jakobsson AH, Sandqvist U, Sjöblom P. The concentration of lidocaine in follicular fluid when used for paracervical block in a human IVF-ET programme. *Hum Reprod*. 1990;5(8):920–3. doi: [10.1093/oxfordjournals.humrep.a137220](https://doi.org/10.1093/oxfordjournals.humrep.a137220).
73. Schnell VL, Sacco AG, Savoy-Moore RT, Ataya KM, Moghissi KS. Effects of oocyte exposure to local anesthetics on in vitro fertilization and embryo development in the mouse. *Reprod Toxicol*. 1992;6(4):323–7. doi: [10.1016/0890-6238\(92\)90195-y](https://doi.org/10.1016/0890-6238(92)90195-y).
74. Andersson S, Lundeberg T. Acupuncture—from empiricism to science: Functional background to acupuncture effects in pain and disease. *Med Hypotheses*. 1995;45(3):271–81. doi: [10.1016/0306-9877\(95\)90117-5](https://doi.org/10.1016/0306-9877(95)90117-5).
75. Manheimer E, White A, Berman B, Forys K, Ernst E. Meta-analysis: Acupuncture for low back pain [published correction appears in Ann Intern Med. 2005 Jun 7;142(11):950–1]. *Ann Intern Med*. 2005;142(8):651–3. doi: [10.7326/0003-4819-142-8-200504190-00014](https://doi.org/10.7326/0003-4819-142-8-200504190-00014).
76. Wu MT, Sheen JM, Chuang KH, et al. Neuronal specificity of acupuncture response: A fMRI study with electroacupuncture. *Neuroimage*. 2002;16(4):1028–37. doi: [10.1006/nimg.2002.1145](https://doi.org/10.1006/nimg.2002.1145).
77. Han JS. Acupuncture: Neuropeptide release produced by electrical stimulation of different frequencies. *Trends Neurosci*. 2003;26(1):17–22. doi: [10.1016/s0166-2236\(02\)00006-1](https://doi.org/10.1016/s0166-2236(02)00006-1).
78. Stener-Victorin E, Waldenström U, Andersson SA, Wikland M. Reduction of blood flow impedance in the uterine arteries of infertile women with electro-acupuncture. *Hum Reprod*. 1996;11(6):1314–7. doi: [10.1093/oxfordjournals.humrep.a019378](https://doi.org/10.1093/oxfordjournals.humrep.a019378).
79. Kim J, Shin KH, Na CS. Effect of acupuncture treatment on uterine motility and cyclooxygenase-2 expression in pregnant rats. *Gynecol Obstet Invest*. 2000;50(4):225–30. doi: [10.1159/000010321](https://doi.org/10.1159/000010321).
80. Manheimer E, Zhang G, Udooff L, et al. Effects of acupuncture on rates of pregnancy and live birth among women undergoing in vitro fertilisation: Systematic review and meta-analysis. *BMJ*. 2008;336(7643):545–9. doi: [10.1136/bmj.39471.430451.BE](https://doi.org/10.1136/bmj.39471.430451.BE).
81. El-Toukhy T, Sunkara SK, Khairy M, Dyer R, Khalaf Y, Coomarasamy A. A systematic review and meta-analysis of acupuncture in in vitro fertilisation. *BJOG*. 2008;115(10):1203–13. doi: [10.1111/j.1471-0528.2008.01838.x](https://doi.org/10.1111/j.1471-0528.2008.01838.x).
82. Cheong Y, Nardo LG, Rutherford T, Ledger W. Acupuncture and herbal medicine in in vitro fertilisation: A review of the evidence for clinical practice. *Hum Fertil (Camb)*. 2010;13(1):3–12. doi: [10.3109/14647270903438830](https://doi.org/10.3109/14647270903438830).
83. Xie ZY, Peng ZH, Yao B, et al. The effects of acupuncture on pregnancy outcomes of in vitro fertilization: A systematic review and meta-analysis. *BMC Complement Altern Med*. 2019;19(1):131. doi: [10.1186/s12906-019-2523-7](https://doi.org/10.1186/s12906-019-2523-7)

84. Smith CA, de Lacey S, Chapman M, et al. Effect of acupuncture vs sham acupuncture on live births among women undergoing in vitro fertilization: A randomized clinical trial. *JAMA*. 2018;319(19):1990–8. doi: [10.1001/jama.2018.5336](https://doi.org/10.1001/jama.2018.5336).
85. Simpson JL, Kuliev A, Rechitsky S. Overview of preimplantation genetic diagnosis (PGD): Historical perspective and future direction. *Methods Mol Biol*. 2019;1885:23–43. doi: [10.1007/978-1-4939-8889-1_2](https://doi.org/10.1007/978-1-4939-8889-1_2).
86. Child AH, Aragon-Martin JA, Sage K. Genetic testing in Marfan syndrome. *Br J Hosp Med (Lond)*. 2016;77(1):38–41. doi: [10.12968/hmed.2016.77.1.38](https://doi.org/10.12968/hmed.2016.77.1.38).
87. Sciorio R, Aiello R, Irollo AM. Review: Preimplantation genetic diagnosis (PGD) as a reproductive option in patients with neurodegenerative disorders. *Reprod Biol*. 2021;21(1):100468. doi: [10.1016/j.repbio.2020.100468](https://doi.org/10.1016/j.repbio.2020.100468).
88. Shatalin D, Gozal Y, Grisaru-Granovsky S, Ioscovich A. Five years' experience in an anesthesiology antenatal clinic for high-risk patients. *J Perinat Med*. 2018;46(3):287–91. doi: [10.1515/jpm-2017-0016](https://doi.org/10.1515/jpm-2017-0016).
89. Fitzgerald M, Donal Ryan D. Cystic fibrosis and anaesthesia. *Continuing Education in Anaesthesia Critical Care & Pain*. 2011;11(6):204–9.
90. McNally EM, Mann DL, Pinto Y, et al. Clinical care recommendations for cardiologists treating adults with myotonic dystrophy. *J Am Heart Assoc*. 2020;9(4):e014006. doi: [10.1161/JAHA.119.014006](https://doi.org/10.1161/JAHA.119.014006).
91. Murugappan G, Alvero RJ, Lyell DJ, Khandelwal A, Leonard SA. Development and validation of a risk prediction index for severe maternal morbidity based on preconception comorbidities among infertile patients. *Fertil Steril*. 2021;116(5):1372–80. doi: [10.1016/j.fertnstert.2021.06.024](https://doi.org/10.1016/j.fertnstert.2021.06.024).

Introduction

Endometriosis, as a clinical entity, has been recognized and intensely investigated for well over 100 years. Despite the accumulation of an enormous amount of information, uncertainty still exists regarding aetiologies, clinical consequences, and treatment efficacy. The two most common complaints leading to a diagnosis of endometriosis are pelvic pain and infertility. Medical, surgical, or a combination of both approaches have been employed to improve many of the symptoms associated with endometriosis. Assisted reproduction technology (ART) has also become an indispensable asset in providing affected couples with viable pregnancies. There is also a growing body of data demonstrating the effectiveness of GnRH agonists and laparoscopically guided laser ablation in increasing live birth rates.

Endometriosis and infertility

There is little debate that the extensive anatomical distortion and tubal obstruction frequently attributed to severe endometriosis does impair fertility. Less clear is the reported association between minimal or mild endometriosis and infertility in the absence of any mechanical disruption. Although there is no conclusive evidence that minimal to moderate endometriosis actually causes infertility, several studies dating back to the 1930s have suggested that there is at least an association between the two [1]. In the 1970s, three studies retrospectively compared the incidence of endometriosis in women undergoing laparoscopy for infertility or voluntary sterilization [2–4]. The incidences of endometriosis ranged from 21% to 48% in infertile women, while endometriosis was noted in only 1.3%–5% of fertile women undergoing tubal ligation. Subsequent studies [5, 6] including one prospective investigation [7], have demonstrated that among women undergoing insemination with donor sperm due to severe male factor infertility, those with coexisting endometriosis had markedly fewer conceptions per exposure than women who did not have the disease. Another prospective double-blind study [8], which looked specifically at women with mild endometriosis compared to women without endometriosis, was able to show a trend towards higher pregnancy rates in women without the disease. The results, however, did not reach statistical significance. This may be attributable to the fact that the number of patients enrolled did not meet the study's power calculation.

Although the aforementioned studies were methodologically imperfect and far from conclusive, virtually every area within the reproductive process has been intensely investigated in an attempt to describe a causal relationship between endometriosis and infertility. The results of several tangential lines of investigation have added to the confusion, as studies are frequently in direct contradiction to one another. Investigators have suggested that women with mild to moderate endometriosis have a higher incidence of endocrine abnormalities [9], anovulation [10], corpus

luteum insufficiency [11], hyperprolactinemia [12], luteinized unruptured follicle syndrome [13], and spontaneous abortions [14]. However, other well-organized, prospective studies have found most of these factors to be either normal or lacking in clinical significance [15–20].

Immune dysfunction in endometriosis has become the focus of more recent efforts, as it is hypothesized that immunity plays a role in the pathogenesis of the disease. Several immunologic abnormalities, which could potentially impair fertility, have been identified. Researchers have reported increased B cell activity, with the production of specific antibodies against endometrial antigens, T cell and macrophage dysfunction, and nonspecific polyclonal B cell activation, which may negatively impact implantation [15, 21]. There has been evidence to suggest peritoneal fluid in patients suffering from endometriosis may be compromised by inflammatory mediators, which may negatively impact the fertilization of released oocytes [22]. Recent evidence has shown that these cytokines and eicosanoids may impact sperm motility [23], sperm function [24], and even interaction between sperm and oocyte [25]. As with other factors, many conflicting reports have emerged. Furthermore, it is not at all clear which is the cause and which the effect, or what role each abnormality actually plays in the pathogenesis of endometriosis-associated infertility.

Many investigators have proposed that endometriosis is actually caused by interplay between environmental and genetic factors. Many have also suggested that certain genetic polymorphisms associated with endometriosis could predispose a woman to infertility. In a review of the advances in the genetics of endometriosis, Dun et al. [26] reviewed the most commonly studied genes thought to be associated with endometriosis. More than 18 genes were implicated, with most relating to xenobiotic metabolism, steroid action and receptors, and inflammatory and angiogenic factors. A direct association between these genetic polymorphisms and endometriosis-associated infertility has yet to be shown though. More recently, research has focused on microRNA as a tool to aid in the diagnosis of endometriosis. One study utilized next generation sequencing to identify a panel of microRNA associated with endometriosis. This panel allowed endometriosis to be diagnosed with a blood sample with comparable sensitivity to laparoscopy [27]. Another study used microRNA in peritoneal fluid as a biomarker to predict infertility in patients with endometriosis [28].

Previous studies using magnetic resonance imaging of the uterus in patients with endometriosis have demonstrated up to a 90% prevalence rate of adenomyotic lesions in those patients with established pelvic endometriosis. This association between endometriosis and adenomyosis may also contribute to the infertility seen in these patients, particularly those with severe disease [29].

As stated, one argument that has been proposed against a causal relationship between endometriosis and infertility is the outright failure of medical or surgical treatment to significantly improve pregnancy success in these patients. The use of certain medical

treatments, otherwise successful in alleviating the non-reproductive symptoms of endometriosis, has failed to demonstrate a reasonable improvement in fertility [30]. Earlier studies investigating the effect of surgical ablation of endometriotic lesions, by any one of a number of techniques, have failed to show increased fecundity. One randomized study, however, did show an improved rate of pregnancy for women with minimal/mild endometriosis treated with ablation of endometriotic lesions, when compared with a control group receiving diagnostic laparoscopy alone [31]. However, this study has been criticized for having a lower fecundity rate among untreated patients than would normally be expected, for notifying patients of their treatment status, and for following pregnancies to only 20 weeks. Subsequently, another randomized study, which looked at actual birth rates, failed to demonstrate a reproductive benefit for patients whose lesions were ablated, but had lower power than the first study [32]. When the results were combined, no significant statistical heterogeneity was noted, and the increased chance of achieving pregnancy after surgery was found to be only 8.6% (95% confidence interval [CI] 2.1%–15.0%) [33].

However, surgical techniques for endometriosis have diversified and improved in the past two decades. Nevertheless, the 2017 NIH NICE guidelines [34] demonstrate there is minimal evidence to suggest that surgical treatment for endometriosis improves outcomes compared to diagnostic laparoscopy alone.

With regard to endometriomas, previous studies did not find a benefit to surgical intervention. A Cochrane review of four trials concluded that surgery (aspiration or cystectomy) versus expectant management showed no evidence of a benefit for clinical pregnancy with either technique [35]. Another meta-analysis had similar findings, demonstrating that surgical treatment of endometrioma did not alter the outcome of IVF/ICSI treatment [36]. However, surgical techniques have again advanced and include endometrioma excision, stripping, plasma energy ablation, and CO₂ vaporization. A recent review focusing on studies from 2015 to 2019 noted increased pregnancy rates from 20%–60% for patients who underwent surgical intervention of endometriomas [37]. In a recent meta-analysis of 553 women with endometriomas, four treatment groups were evaluated: surgery and ART, surgery alone, aspiration plus sclerotherapy and ART, and ART alone. ART alone had the lowest pregnancy rate (32%, CI: 15.0–52.0, $p = 0.02$), whereas surgery alone had the highest pregnancy rate (43.8%, CI: 22.5–66.4, $p = 0.01$) [38].

Ovulation induction and insemination

Controlled ovarian stimulation (COS), in combination with intrauterine insemination (IUI), has proven to be a cost-effective and appropriate first-line treatment for many infertility diagnoses [39]. However, the data does not suggest that this approach may be as effective for patients with endometriosis. Deaton et al. [40] demonstrated increased fecundity in patients treated with clomiphene citrate and IUI who had already undergone surgical treatment, but fecundity was still low at 9.2%. However, Fedele et al. [41] reported that the increased conception rate with COS and IUI did not lead to a significantly different pregnancy rate at six months. Furthermore, a retrospective comparison of COS and IUI reported per-cycle pregnancy rates of 6.5%, 11.8%, and 15.3% for endometriosis, male factor, and unexplained infertility, respectively [42]. Similarly, although with more optimistic results, a prospective, observational study reported pregnancy rates of 16.3% and 33.6% following COS/IUI in patients with

endometriosis and unexplained infertility, respectively [43]. In a meta-analysis, Hughes [44] reported that a diagnosis of endometriosis decreased the per-cycle COS/IUI conception rate by half. Also, a later prospective, randomized study reported live birth rates of 11% and 2% for endometriosis patients undergoing COS/IUI and no treatment, respectively [45]. While this demonstrates a live birth odds ratio (OR) of 5.5 for the treatment group, the actual percentage of live births after treatment remains relatively low. Failure of COS/IUI has been correlated with advanced endometriosis. A retrospective study of 92 patients found that more than a third of patients failing to conceive after four ovulatory cycles of clomiphene citrate had stage III or IV disease, an endometrioma, pelvic adhesions, and/or tubal disease [46]. However, a retrospective, controlled cohort study of 259 COS/IUI cycles found no difference in cycle pregnancy rate and cumulative live birth rate between women with surgically treated minimal to mild endometriosis and women with unexplained infertility, indicating potentially improved outcomes after surgical treatment [47].

The advent of aromatase inhibitors added to the armamentarium of therapeutic modalities for the treatment of endometriosis. Wu et al. [48] found that a third-generation aromatase inhibitor was able to achieve a reasonable pregnancy rate, with a thicker endometrium but fewer ovulatory follicles, when randomized and compared with clomiphene citrate. However, a study by Abu Hashim et al. showed no significant difference in the clinical pregnancy rate per cycle in groups randomized to receive either letrozole (a third-generation aromatase inhibitor) or clomiphene citrate for controlled ovarian hyperstimulation (15.9% for letrozole and 14.5% for clomiphene citrate) [49].

The use of GnRH antagonists in IUI cycles with COS has also been studied. A randomized, double-blinded, placebo-controlled trial showed no difference in live birth rates for women with minimal or mild endometriosis when comparing women who were treated with GnRH antagonist to those who received a placebo [50]. Another randomized controlled trial (RCT) demonstrated no significant difference in pregnancy rate between a group receiving letrozole for two months, triptorelin for two months, and a control group with no medication. There was no difference in pregnancy rate amongst the three groups [51].

Endometriosis and ART

Treatment strategies for the infertile couple must be based on the specific situation. For young women with only minimal or mild endometriosis, expectant management may be the most appropriate course. However, for women approaching the end of their reproductive age, the chances of conceiving drop precipitously. In these women, intervention, in the form of COS/IUI or in vitro fertilization (IVF), may be warranted more expeditiously [52]. The lower cost and low complication rate of ovulation induction and IUI make the combination an attractive first step. However, for women with severe endometriosis or tubal disease, or when male factor or a combination of aetiologies is involved, assisted reproduction such as IVF may be pursued sooner. In addition, IVF offers the added benefit of being able to directly observe key events in the conception process, such as the assessment of gamete quality, the observation of fertilization, and the evaluation of early embryo development. As a result, the increasing use of ART in the treatment of endometriosis-associated infertility may help to answer some of the questions regarding this elusive association. It is thought that the use of IVF–embryo transfer (ET) in the infertile patient with endometriosis removes critical steps

in reproduction, such as fertilization and early embryo development, from an *in vivo* environment that some have suggested is hostile to these processes. A review of clinical and biological studies described multiple markers of decreased oocyte quality retrieved from women with endometriosis, including altered morphology, decreased cytoplasmic mitochondrial content, higher failure rate of *in vitro* maturation, reduced retrieval of mature oocytes, and decreased fertilization rate [53]. However, they hypothesized that while fewer high-quality embryos would be available, that IVF-ART may be able to overcome this issue and have similar pregnancy outcomes. Earlier studies contradicted this theory, showing lower pregnancy rates, particularly in women with moderate to severe disease [54, 55]. However, a recent study compared IVF pregnancy outcomes in patients with endometriosis, male factor infertility, and single gene disorders. Pre-implantation genetic testing for aneuploidy was performed for all embryos before transfer. Endometriosis had comparable aneuploidy to male factor infertility, and there were no differences in clinical pregnancy, miscarriage, or live birth rate amongst the three groups [56].

Technology has advanced and a recent study has shown continued support for the study by Abu Hashim. In 2019, Se Jeong Kim et al. conducted a retrospective cohort study for women with endometriosis undergoing IVF. The study sought to examine the effects of letrozole on these women undergoing ovarian stimulation for IVF. Kim used two separate protocols in order to conduct this study. The first protocol involved combination therapy consisting of letrozole and gonadotropins, whereas the second protocol utilized conventional IVF with gonadotropins. The results show that patients receiving the first protocol with letrozole resulted in significantly lower peak oestradiol levels in IVF than with those that received the second protocol. Protocol one's patients had a lower mean percentage of mature oocytes than protocol two patients. Despite maintaining low oestrogen levels, there was no significant difference in oocyte and embryo yield between the two groups [57].

Certainly, the development of GnRH agonists and transvaginal oocyte retrieval has been associated with increased success in the use of IVF for endometriosis-associated infertility. However, the value of reported ART results must be considered along with the understanding that there is great clinical and laboratory variability among centres, leading to a wide range of reported pregnancy rates. Furthermore, most studies are retrospective and observational and are therefore of limited value in reaching definitive conclusions regarding therapy efficacy. Barnhart et al. [58] performed a meta-analysis on the studies evaluating the effects of endometriosis on the outcomes of ARTs. They evaluated a total of 22 articles and concluded that, overall, patients with endometriosis had lower pregnancy rates, decreased fertilization and implantation rates, and a decreased number of oocytes retrieved compared to controls of tubal factor infertility. Another meta-analysis looking at the association between endometriosis and ART outcomes, which assessed results from 36 studies, found that when compared to women without endometriosis, those with the disease had lower clinical pregnancy rates per patient (OR 0.78, 95% CI 0.65–0.94) and lower mean numbers of oocytes retrieved per cycle (mean difference –1.98, 95% CI –2.87 to –1.09); however, they found similar live birth rates (OR 0.94, 95% CI 0.84–1.06) and therefore comparable success with IVF/intracytoplasmic sperm injection (ICSI) [59].

In a review article on the treatment of infertility associated with deep endometriosis, the authors looked at six studies that

investigated the outcomes of IVF in patients with severe endometriosis. They found that the pregnancy rate per patient varied between 29% and 68%, with an aggregated rate per patient of 51% (95% CI 45%–56%) [60].

COS and oocyte retrieval

As the practice of assisted reproduction has evolved over the past three decades, so has the efficacy of IVF in the treatment of endometriosis. With regard to the effect of endometriosis on COS and oocyte retrieval, an obvious divide exists between earlier studies using clomiphene citrate with laparoscopic oocyte retrieval and contemporaneous investigations benefiting from the development of GnRH agonists and ultrasound-guided transvaginal oocyte retrieval. Earlier studies reported a reduced oocyte yield in patients with endometriosis undergoing IVF. In one small study, Chillik et al. [61] compared patients with either no endometriosis, mild to moderate endometriosis, or severe disease, and reported that oocyte yield was reduced in those patients of advanced stage. Oehninger et al. [62] reported a similar effect on oocyte retrieval for patients with stage III or IV endometriosis. Both studies suggested that oocyte yield was impaired in this group of patients due to technical difficulties at the time of laparoscopic oocyte retrieval. Alternatively, other researchers have reported decreased folliculogenesis in patients with endometriosis [63–66]. Furthermore, Dlugi et al. [67] and Somigliana et al. [68] reported a significantly lower number of pre-ovulatory follicles in patients with endometriomas when compared to patients with hydrosalpinges. Additionally, another review suggests that endometriomas may have deleterious effects on folliculogenesis and oocyte quality, independent of stretching/mass effect by the cyst [69]. More recently, a prospective trial compared oocyte quality in patients with unilateral endometriomas, patients after surgical treatment for unilateral endometriomas, and tubal factor infertility as a control group. The endometrioma group had a statistically significant increase in immature oocytes (M1s and germinal vesicles) compared to the control group. Additionally, every fourth oocyte from an ovary with an endometrioma had abnormal structural changes [70].

Several studies utilizing GnRH agonists and transvaginal retrieval have not confirmed that endometriosis has a significant effect on oocyte yield. Dmowski et al. [71] retrospectively analysed 237 IVF cycles and found no difference in either folliculogenesis or in the number of oocytes obtained for women with or without endometriosis. In a case-control study comparing 65 cycles of IVF for women with endometriosis to 98 cycles of IVF in patients with tubal infertility, Bergendal et al. [72] found no difference in folliculogenesis or oocyte retrieval. Several recent studies have further concluded that there is no difference in the number of oocytes obtained in patients with mild to moderate endometriosis when compared to patients with more severe disease [73–76]. Barnhart et al. demonstrated a lower number of oocytes retrieved (OR 0.82, 95% CI 0.75–0.90) for patients with endometriosis when compared to patients with tubal factor [58].

The improvement in IVF outcomes brought about by the development of GnRH agonists is largely undisputed. Olivennes et al. [76] reported a significantly improved clinical pregnancy rate for patients treated with GnRH agonists when compared with standard, gonadotropin-only ovarian stimulation protocols. Other investigations have reported similar results [77]. Long-term GnRH agonist suppression has been thought to repress further endometriotic lesions and improve IVF outcome for patients with endometriosis. Dicker and associates [78], as well as Rickes

et al. [79], reported a significantly higher clinical pregnancy rate after six months of GnRH agonist therapy compared with ovarian stimulation with gonadotropins alone. Chedid et al. [80] also investigated the use of a three-month and a three-week GnRH agonist downregulation protocol and reported a significantly increased oocyte yield when compared with controls receiving only gonadotropins. Although they noted an improved pregnancy rate, it did not reach statistical significance.

Nakamura et al. [81] compared GnRH agonist suppression for 60 days with a shorter, mid-luteal downregulation prior to ovulation induction. They reported pregnancy rates of 67% and 27% for the longer and shorter protocols, respectively. Marcus and Edwards [82] also reported a significantly higher pregnancy rate for patients treated with longer GnRH agonist protocols (Table 63.1) [61–64, 71–76, 83–86], although they used different GnRH agonists for the two groups and assigned patients based on their refusal to accept the longer regimen. Surrey et al. [87] investigated a three-month course of GnRH agonist therapy prior to IVF–ET and found the agonist therapy to be associated with a significantly higher ongoing pregnancy rate. Conversely, Chedid et al. [80] found no difference between long and short GnRH agonist administrations. Recently, Kaponis et al. performed a large, multicentre prospective RCT comparing three-month GnRH agonist treatment before IVF attempt compared to no GnRH agonist in women with laparoscopically confirmed and ablated mild endometriosis (ASRM I–II). There was no statistically significant clinical pregnancy rate between the two groups [88]. Surrey responded with the argument that the endometriosis may have already been partially treated with the laparoscopic ablation, and in his prior analysis he had found improved pregnancy rates from GnRH agonists only in patients with stage III–IV disease [89, 90].

The use of continuous oral contraceptive pills prior to assisted reproduction treatment has also been examined. De Ziegler et al. [91] found that six to eight weeks of continuous use of combined oral contraceptive pills before IVF–ET for patients with endometriosis had similar outcomes patients treated with three months of GnRH agonist treatment. A recently published RCT demonstrated no benefit to pregnancy outcomes when using long (three-to-six-month) GnRH agonist protocol compared to 21 days of combined oral contraceptives with five days of GnRH overlap. The study was terminated early, as many women declined to be randomized to the long GnRH group, but preliminary data showed clinical pregnancy rate of 25% (5/20) in the control group, and 20% (4/20) in the ultra-long group ($P > 0.999$; relative risk (RR) 1.25, 95% CI 0.41–3.88). Due to the small sample size, this was not statistically significant. They also found that the control group required fewer days of stimulation and lower total gonadotrophin use, suggesting a better ovarian response than in the long GnRH group [92].

A non-inferiority RCT compared medroxyprogesterone acetate + hMG (human menopausal gonadotropin); dydrogesterone + hMG; and progesterone + hMG prior to IVF–ET. Their primary outcome, oocytes retrieved, was significantly higher in the medroxyprogesterone acetate + hMG group than the two other groups (9.3 ± 5.7 vs 8.0 ± 4.5 vs 7.8 ± 5.2 , $P = 0.021$). Notably, clinical pregnancy and live birth rate were similar amongst all three groups [93]. Another recent RCT evaluating the progestin Dienogest in endometriosis patients for 12 weeks prior to IVF–ET showed decreased antral follicle count, retrieved oocytes, fertilized oocytes, pregnancy rate, and live birth rate [94]. Therefore, the type of progestin used prior to IVF–ET should be chosen judiciously with the available evidence.

TABLE 63.1 Comparison of in Vitro Fertilization–Embryo Transfer Outcomes for Women with and without Endometriosis

Study	Group	Number of Cycles	Clinical Pregnancies (% Cycle)	Study	Group	Number of Cycles	Clinical Pregnancies (% Cycle)
Mahadevan [83]	I–IV	14	14	Inoue [74]	I	111	40
	Tubal	261	10		II	78	42
Wardle [84]	I–IV	17	6		III	51	47
	Tubal	47	11		IV	69	42
Chillik [61]	I/II	10	60		Other	372	44
	III/IV	14	7	Olivennes [76]	I–IV	360	29
Matson [63]	I	24	13		Tubal	160	36
	II	37	14	Geber [73]	I/II	100	29
	III	36	6		III/IV	29	52
	VI	57	2		Tubal	1139	41
	Tubal	40	18	Dmowski [71]	I/II	89	25
Sharma [85]	I/II	135	16		III/IV	30	30
	III/IV	141	8		Other	118	21
	Tubal	994	13	Arici [86]	I/II	43	12
Oehninger [62]	I/II	191	24		III/IV	46	15
	III/IV	35	20		Tubal	147	24
Yovich [64]	I/II	61	13	Bergendal [72]	I–IV	65	28
	III/IV	93	3		Tubal	98	30
	Tubal	49	14	Pal [75]	I/II	45	44
					III/IV	39	33

Recent studies have also analysed the use of GnRH antagonist protocols for IVF in patients with endometriosis. A prospective randomized trial compared GnRH agonist and antagonist protocols for women with mild to moderate endometriosis [95]. This study showed similar implantation and clinical pregnancy rates for patients treated with both GnRH agonist and antagonist protocols. Patients treated with a GnRH agonist, however, had a significantly higher number of additional embryos available for cryopreservation, making the cumulative fecundity rate higher with the agonist protocol.

For now, it appears that endometriosis patients respond to ovarian stimulation in a manner that is similar to other infertility aetiologies. Although standard gonadotropin stimulation protocols work reasonably well, the addition of longer GnRH agonist downregulation or the use of continuous oral contraceptive pills may increase IVF success and should be considered on a case-by-case basis.

Fertilization and early embryo development

It is unclear as to the degree to which endometriosis is a detriment to the process of fertilizing oocytes *in vitro*, as several investigations have now reported significantly impaired fertilization rates for these patients. One early study noted fertilization rates per oocyte of 33%, 63%, and 68% for patients with endometriosis, unexplained infertility, and tubal infertility, respectively [84], whereas another reported a marked impairment in fertilization with the presence of an endometrioma [67]. Bergendal et al. [72] reported fertilization rates of 60% and 78% for patients with endometriosis and tubal factor, respectively ($p < 0.0001$). Other investigators have reported significantly lower fertilization success for stage III or IV endometriosis when compared with stage I or II endometriosis [75, 76]. With regard to early embryo development, researchers have reported fewer embryos reaching the four-cell stage at 48 hours [96], a reduced number of blastomeres at 72 hours [97], and lower cleavage rates when endometriosis is compared with tubal factor or unexplained infertility [98]. Furthermore, Brzek et al. [99] retrospectively analysed video records of 235 embryos and found a statistically significant increase in the incidence of aberrant nuclear and cytoplasmic morphology within embryos from patients with endometriosis.

Conversely, there have been several large studies that have failed to detect an impairment in fertilization. Dmowski et al. [71] analysed 237 cycles and found no difference in either the fertilization rate or the early cleavage rate among patients with endometriosis or tubal factor infertility. Another case-control study, also comparing endometriosis with tubal factor, found no evidence of either impaired fertilization or a decrease in embryo quality [86]. In comparing the effect of progressive endometriosis stages on fertilization and embryo development, Inoue et al. [74] found no differences in either the fertilization rate or the ET rate for 309 patients with stage I–IV endometriosis. Furthermore, Bergendal et al. [72], although reporting impaired fertilization for women with endometriosis, noted no difference in either the cleavage rate or the morphologic embryo score, when compared with tubal infertility.

As it stands, the question of a significant effect by endometriosis on fertilization and *in vitro* embryo development has yet to be answered. Barnhart et al. [58] showed an overall decrease in fertilization rate when all endometriosis patients were compared to patients with tubal infertility, but when stratified by stage of disease, patients with severe endometriosis actually had an increase in fertilization rates. However, more recent studies

have shown that any impaired fertilization has little or no effect on the ultimate outcome of IVF, as pregnancy rates for patients with endometriosis are comparable with other aetiologies. Suzuki et al. [100] found that endometriosis affects oocyte number but not embryo quality or pregnancy outcome, irrespective of the presence of an ovarian endometrioma.

Perhaps the clinical insignificance of impaired fertilization is due to the fact that improved ovarian stimulation and oocyte recovery techniques have led to a surplus of available oocytes for fertilization. An increased oocyte yield can readily sustain a slight decrease in fertilization capacity to produce enough embryos for implantation. This is supported by data regarding ICSI: as expected, fertilization rates increase, but clinical pregnancy rate may not if sufficient embryos are available. Komsky-Elbaz et al. had higher fertilization and day 2 embryo rate with ICSI compared to IVF in sibling oocytes, but the RCT showed no difference in implantation or clinical pregnancy rate [101]. Shebl et al. matched IVF and ICSI in patients with endometriosis to IVF and ICSI to endometriosis-free patients. While the endometriosis group had lower retrieved MII and significantly reduced fertilization with conventional IVF, clinical pregnancy, miscarriage, and live birth rate were similar between the groups [102].

Endometriosis does not show an impact on the euploid blastocyst rate as well. More specifically, in a case-controlled study conducted by Alberto Vaiarelli et al., there was no impact on the blastocyst rate per cohort of inseminated metaphase-II oocytes. In this study, patients who were diagnosed via surgery were matched to two controls: maternal age during retrieval, number of previous failed IVF treatments and number of metaphase-II oocytes retrieved. Results show identical mean euploid blastocyst rates, as well as similar vitrified-warmed single euploid blastocyst transfer live birth rates for both matched controls. The importance of this study is thus that endometriosis might not directly impair oocyte developmental and reproductive competence. Nonetheless, the potential impact on metaphase-II oocytes retrieved cannot be disregarded [103].

Implantation, pregnancy, and loss

Assuming a minimum number of good-quality embryos are available for transfer, a successful live birth is dependent on adequate implantation and a low rate of spontaneous abortion. However, as a result of the transfer of multiple embryos, a lower rate of implantation has not necessarily translated into a low pregnancy rate. Although a few contemporary studies have in fact reported reduced implantation rates, most have failed to demonstrate a correspondingly low pregnancy rate for patients with endometriosis. Some early studies have shown a decrease in the implantation rate with a subsequent decrease in the pregnancy rate [62, 63, 83]. In a small study, Chilliak et al. [61] reported a significantly lower implantation and pregnancy rate for patients with stage III or IV endometriosis when compared to patients with tubal factor or endometriosis of a lesser severity. Matson and Yovich [63] demonstrated pregnancy rates of 18%, 13%, 14%, 6%, and 2%, for patients with tubal factor and stage I–IV endometriosis, respectively. In a case-control study of 284 IVF cycles, Arici et al. [86] reported a significantly lower implantation rate of 3.9% for patients with endometriosis compared with 8.1% and 7.2% for tubal infertility and unexplained infertility, respectively. They also demonstrated a trend towards a lower pregnancy rate in patients with endometriosis, although this did not reach significance. More recent studies have taken this finding and added live birth and cumulative pregnancy rates. Omland et al. [55]

found the live birth rate after transfer of two embryos to be 66.0% compared with 78.8% for unexplained aetiology of infertility. Kuivasaari et al. [54] found a significantly lower cumulative pregnancy rate after one to four IVF/ICSI treatments in women with stage III/IV endometriosis compared to women with stage I/II endometriosis and a control group of women with tubal infertility. However, the recent studies by Komsky-Elbaz [101] and Shebl et al. [102] showed similar implantation rates and clinical pregnancy rates.

Errors in implantation may be attributed to the relationship between endometriosis and adenomyosis. Recent studies have suggested that treatment with either prolonged downregulation with GnRH agonists [104] or oral contraceptives [91] may help overcome the effects of adenomyosis on the endometrium. A recent systematic review found a fourfold increase in the odds of clinic pregnancy (OR 4.28, 95% CI 2.00–9.15) with administration of GnRH agonists for a period of three to six months prior to IVF in patients with endometriosis [105]; however, the safety data from this analysis were not readily available.

A competitive vasopressin/oxytocin receptor antagonist, atosiban, is undergoing evaluation for utility in treating endometriosis-associated pain and infertility. Endometrial cells express oxytocin receptors (OTRs), that have the capacity to trigger the production of prostaglandin (PG)F2a and E2 when oxytocin binds [106]. Both endometriotic and adenomyotic endometrial cells have increased levels of prostaglandins [107, 108]. Decreased endometrial prostaglandin expression may make a more favourable environment for implantation. Atosiban demonstrated a higher pregnancy rate per cycle (58.3% atosiban vs 38.3% control group) when administered before frozen embryo transfer [109].

While Simon et al. [110] also reported lower implantation and pregnancy rates for patients with endometriosis versus tubal infertility, they added a dimension to the data by analysing the outcomes of oocyte donation from donors with and without endometriosis. They reported comparable implantation and pregnancy rates for women with and without endometriosis who received oocytes from donors without endometriosis. However, patients who received oocytes from endometriotic ovaries had significantly lower implantation rates. Another study reported on 239 oocyte donor cycles and found that the presence of endometriosis in the recipient had no effect on implantation or pregnancy rates, regardless of the disease stage [111]. From this, it has been suggested that an endometriosis-associated impairment of implantation results from a compromise to the potential of the oocyte or early embryo, and not to the endometrium itself.

Furthermore, a September 2020 study conducted by Bishop et al. [56] demonstrates that endometriosis does not impact live

birth rates in frozen embryo transfers of euploid blastocysts. This was a multicentre and retrospective cohort study including all patients undergoing euploid frozen blastocyst transfer. Analysis of 459 euploid frozen embryo transfer cycles among 328 unique patients showed that there was no difference in clinical pregnancy, pregnancy loss, or live birth rates in patients with endometriosis compared with non-infertile patients who underwent assisted reproduction to screen embryos and couples with isolated male factor infertility. For those who have undergone pre-implantation testing, aneuploidy rates were lowest, whereas endometriosis patients had similar aneuploidy rates when compared with male infertility factored patients. They concluded that by controlling embryo quality using frozen blastocysts, endometriosis compared with male infertility and non-infertile patients resulted in similar pregnancy outcomes.

A matched case-control study was performed comparing the implantation rates for patients with stage III/IV endometriosis with those of women who are free of the disease. Transfers using matched sibling oocytes from the same donor demonstrated no statistically significant difference in pregnancy, implantation, miscarriage, and live rates [112]. This study suggests that endometrial receptivity and subsequent implantation rate may not be affected by stage III/IV endometriosis.

Several large investigations have failed to demonstrate either an impaired implantation rate or a lower pregnancy rate for patients with endometriosis when comparing stage by stage or with other infertility aetiologies [72–76]. Geber et al. [73] reported pregnancy rates in 140 cycles of 40% and 45% for patients with endometriosis or tubal infertility, respectively. Olivennes et al. noted similar pregnancy rates of 29% for endometriosis and 36% for tubal factor [76], while another study reported rates of 28% and 30%, respectively [72]. In a study of 681 women with and without endometriosis, Inoue et al. [74] found no difference in the IVF conception rate between the two groups. Several comparisons within endometriosis stages have reported similar pregnancy rates despite increasing disease severity [62, 71, 73, 86]. Pal et al. analysed IVF cycles in endometriosis patients with either stage I/II or stage III/IV disease. Although they reported a lower fertilization rate for patients with stage III or IV endometriosis, clinical pregnancy rates did not differ significantly between the two groups [75]. In their meta-analysis, Barnhart et al. [58] calculated that the adjusted OR of achieving pregnancy compared with the group of controls was 0.56, 0.79, and 0.46, respectively, for overall patients, stage I/II patients, and stage III/IV patients, respectively (Table 63.2).

A few studies have associated endometriosis with increased pregnancy loss during IVF cycles. Oehninger et al. [62] noted a

TABLE 63.2 Results of Bivariate Analysis and Multiple Logistic Regression Comparing Endometriosis Patients with Stage III/IV Disease with Patients with Stage I/II Disease

Outcome	Endometriosis Stage III/IV	Endometriosis Stage I/II	p-value	Crude OR (95% CI)	Adjusted OR ^a (95% CI)
Pregnancy rate	13.84	21.12	<0.001	0.60 (0.42–0.87)	0.64 (0.34–1.17)
Fertilization rate	74.47	58.38	<0.001	1.11 (1.09–1.13)	Not interpretable
Implantation rate	10.23	11.31	0.003	0.93 (0.89–0.98)	0.21 (0.15–0.32)
Mean oocyte count	6.70	8.19	<0.001	0.83 (0.78–0.87)	0.31 (0.24–0.39)
Peak E2	1447.74	5813.38	<0.001	N/A	N/A

Source: Barnhart et al. (2002), with permission. [58]

Note: Total number of observations: 699.

^a Adjusted for publication date and age.

Abbreviations: CI, confidence interval; E2, oestradiol; N/A, not applicable; OR, odds ratio.

higher miscarriage rate following IVF among patients with stage III or IV endometriosis when compared to those with less severe disease. Along with a diminished oocyte yield and poor embryo quality, Yanushpolsky et al. [96] reported a significantly higher early pregnancy loss when endometriomas were aspirated at the time of oocyte retrieval. However, another large study comparing patients with aspirated endometriomas to others with endometriosis found no difference in oocyte yield, embryo quality, pregnancy rate, or miscarriage [113]. Furthermore, most studies have not reported a significant endometriosis-associated increase in pregnancy loss [72, 73].

Endometriosis may also be associated with late pregnancy complications, such as preterm birth. Stephansson et al. [114] showed that, compared with women without endometriosis, women with endometriosis had a higher risk of preterm birth, with an adjusted OR of 1.33. Conversely, Fernando et al. [115] showed an increased risk of preterm birth only when endometrioma was present. Women with endometriosis, but without endometrioma, did not show an increased risk for preterm birth when compared to women without endometriosis. A more recent meta-analysis calculated an adjusted OR of 1.70 for preterm birth in patients with endometriosis, and cited 17 other studies reporting significantly increased incidence of preterm birth [116]. Another observational study of 196,722 pregnancies reported a RR of 1.16 of preterm birth for patients with endometriosis, and a RR of 1.40 for miscarriage [117].

Surgery and ART

As stated earlier, data are promising for surgery as an isolated treatment for endometriosis-related infertility. However, it is less clear what the effect of surgery for endometriosis is on ART outcome. Unfortunately, there have been no prospective, randomized studies.

One retrospective study compared IVF with repeat surgery for patients with stage III or IV endometriosis [118]. A Cochrane review of two randomized trials comparing the effectiveness of laparoscopic surgery in the treatment of subfertility associated with endometriosis versus other treatment modalities or placebo found that use of laparoscopic surgery may improve the chance of pregnancy by an OR of 1.6 [119]. Pregnancy rates were reported as 70% over two cycles of IVF, compared with 24% for the nine months following surgery. There are no similar randomized studies evaluating the effects of surgery on severe disease. A non-randomized study [33] demonstrated that the cumulative probability of pregnancy in 216 infertile patients with severe disease two years after surgery was significantly increased.

In another study, Garcia-Velasco et al. reported no difference in fertilization, implantation, or pregnancy rates for patients who had undergone removal of an endometrioma, as compared to patients with suspected endometriomas that were not removed [120]. A meta-analysis [121] of five studies agreed with these results by concluding that surgical management of endometriomas has no significant effect on IVF pregnancy rates and ovarian response to stimulation compared with no treatment.

In another randomized study comparing patients with and without varying degrees of endometriosis undergoing ICSI for male factor infertility found no difference in either fertilization or pregnancy and implantation rates between women with and without endometriosis, although significantly fewer oocytes were retrieved from patients with endometriosis [122].

However, a study by Bianchi et al. of women with deep infiltrative endometriosis found that extensive laparoscopic excision

of endometriotic lesions improved pregnancy outcomes significantly (OR 2.45) [123]. Until better data are available, however, no definitive conclusions can be drawn regarding the role of surgery for endometriosis prior to ART. In fact, one study [124] found that in the absence of tubal occlusion or severe male factor infertility, laparoscopy may still be considered for the treatment of endometriosis even after multiple failed IVF cycles.

Future directions

Some researchers have suggested that endometriosis is associated with impaired folliculogenesis and a decreased oocyte yield. Although the data are conflicting, it is possible that the introduction of aromatase inhibitors may represent another large step forward in improving ovarian stimulation protocols and increasing IVF success. Further study of GnRH antagonists may also show a benefit for patients with endometriosis. Furthermore, the use of donor oocytes has been suggested to improve efficacy in patients with endometriosis. As COS protocols become more tolerable and as oocyte cryopreservation becomes efficacious and efficient, it is possible that an increased number of women with endometriosis who have failed standard IVF will benefit from donation.

There is evidence for and against an endometriosis-associated impairment of oocyte fertilization *in vitro*. One of the tremendous benefits of fertilizing an oocyte *in vitro* is the ability to assess the process on a case-by-case basis. For patients with endometriosis who are experiencing fertilization difficulty, ICSI is a valuable addition to the technology of assisted reproduction for this disease. Indeed, ICSI has proven to be of tremendous worth in achieving pregnancy in couples with male factor infertility. Minguez et al. [122] analysed 980 cycles of ICSI for couples with male factor infertility, of which 101 cycles were also complicated by endometriosis. They found no significant difference in fertilization, implantation, or pregnancy rates with coexisting endometriosis. Finally, there is an increasing interest in the prolongation of *in vitro* embryo culture, with many investigators studying the efficacy of blastocyst development and implantation. An endometriosis-associated detriment to implantation may be responsible for some IVF failures. Although reports are conflicting, some have suggested an impaired early embryo development in patients with endometriosis. It is possible that the practice of culture to the blastocyst stage in these patients may allow for the transfer of a more selected group of healthier embryos, thus improving the implantation rate. Furthermore, the adoption of various techniques in embryo manipulation, such as assisted hatching, may also have a positive effect on the implantation rate for these patients. One RCT did show improved implantation rates with laser assisted hatching (OR 1.86, CI: 1.24–2.80, $p = 0.002$), but more studies will be needed [125].

As pre-implantation genetic testing for aneuploidy (PGT-A) increases in popularity as a means of assessing quality of embryos prior to transfer, it is reasonable to assume that the transfer of embryos that have been selected as being chromosomally normal will lead to an increase in the success of IVF in patients with endometriosis.

Additional successes in pregnancy for patients diagnosed with endometriosis might be attributed to metformin. Metformin is originally an insulin sensitizer widely used to treat type 2 diabetes mellitus; however, it could be used as treatment for endometriosis without serious side effects because metformin simply increases the activity of superoxide dismutase and decreases the levels of the vascular endothelial growth factor. A systematic review of metformin studies for endometriosis from Stochino-Loi

et al. [126] highlights its costs and benefits. According to this review, medical treatments such as fulguration or excision may help endometriosis pain and the progression of endometriosis lesions, but the adverse effects can drastically compromise ability to conceive. On the other hand, metformin seems to have therapeutic potential, acting as an anti-inflammatory and antiproliferative agent. Another study conducted by Kimber-Trojnar et al. [127] also found similar results. Nonetheless, more research must be conducted to further the use and potential of metformin.

Other frontiers include a non-invasive diagnostic marker for endometriosis. B-Cell Lymphoma 6, also known as BCL6, and Sirtuin 1, or SIRT 1, are these potential biomarkers. In a study conducted by Sansone et al. [128], BCL6 and SIRT1 are measured in 20 women diagnosed with endometriosis (ten stage I/II and ten stage III/IV) using enzyme-linked immunoassay (ELISA). Although results show that higher levels of SIRT1 were found in advanced stages of endometriosis compared to controls and lower stages of endometriosis, there were no significant differences between BCL6 and SIRT1 in other bodily fluids. BCL6 and SIRT1 have a large potential to be non-invasive markers to diagnose endometriosis. The potential for these two markers should thus be further studied and researched to assess outcomes of treatment after diagnosis with these biomarkers and consequently simplify the diagnosis of endometriosis.

Finally, advances in surgical techniques may allow for improved surgical management of infertility associated endometriosis, and as ease and efficiency improve, increase the feasibility of performing RCTs involving surgical interventions. The Laparoscopic versus Robotic Surgery for Endometriosis (LAROSE) trial has already demonstrated similar outcomes between robotic and laparoscopic surgery in terms of operative time, complications, and quality of life at six weeks and six months [129]. Currently, no data is available for robotic surgery outcomes on endometriosis-related fertility, but the increased visibility and dexterity may allow for more comprehensive treatment and less detriment to ovarian reserve when treating endometriomas.

Conclusion

It is important to stress the heterogeneous nature of the data that have been reviewed. Laboratory and clinical practices vary greatly from centre to centre, as do the corresponding IVF success rates. Randomized, prospective studies designed to answer key questions about the optimum algorithmic approach to the treatment of endometriosis-associated infertility simply do not exist.

Although ART procedure alterations are site specific, the vast majority of endometriosis patients undergo the same treatment protocol as for those patients with tubal factor or unexplained infertility. There is, to date, no compelling evidence that endometriosis patients benefit from significant alterations from standard ART protocols or procedures. The data to date show mixed evidence of prolonged GnRH agonist downregulation. Until large, randomized, prospective studies have answered questions regarding the optimum length of downregulation, the use of *in vitro* maturation or manipulation, the role of autoantibodies and immunosuppression, and other controversies, it is likely that patients with endometriosis will continue to undergo similar treatment protocol as all-comers.

At the very least, it can be said that ART represents a tremendous advancement for women who, for whatever reason, have been unable to achieve pregnancy. ART may be the best option to treat patients with endometriosis but with the development of

metformin, elagolix, BCL6, and SIRT1 comes promising advancements. Future research should seek to pursue the effects of such developments in order to better address the fertility issues that arise through endometriosis. Nonetheless, it is important to consider surgery to be a reliable source of diagnosis until further research. If ART truly is the most reliable way to treat endometriosis, then efforts should be focused on enhancing ART to ultimately improve euploidy rates and birth rates. For the patient with endometriosis, evolving options in pharmacotherapy and assisted reproduction may finally offer the prospect of a pain-free and reproductive life.

References

1. Counsellor VS. Endometriosis: A clinical and surgical review. Am J Obstet Gynecol. 1938;36:877–88.
2. Hasson HM. Incidence of endometriosis in diagnostic laparoscopy. J Reprod Med. 1976;16:135–8.
3. Strathy JH, Molgaard CA, Coulam CB, Melton LJ. Endometriosis and infertility: A laparoscopic study of endometriosis among fertile and infertile women. Fertil Steril. 1982;38:667–72.
4. Drake TS, Grunert GM. The unsuspected pelvic factor in the infertility investigation. Fertil Steril. 1980;34:27–31.
5. Hammond MG, Jordan S, Sloan CS. Factors affecting pregnancy rates in a donor insemination program using frozen semen. Am J Obstet Gynecol. 1986;155:480–5.
6. Yeh J, Seibel MM. Artificial insemination with donor sperm: A review of 108 patients. Obstet Gynecol. 1987;70:313–6.
7. Jansen RPS. Minimal endometriosis and reduced fecundability: Prospective evidence from an artificial insemination by a donor program. Fertil Steril. 1986;46:141–3.
8. Matorras R, Corcostegui B, Esteban J, et al. Fertility in women with minimal endometriosis compared with normal women was assessed by means of a donor insemination program in unstimulated cycles. Am J Obstet Gynecol. 2010;203:345.e1–6.
9. Bancroft K, Vaughan-Williams CA, Elstein M. Pituitary–ovarian function in women with minimal or mild endometriosis and otherwise unexplained infertility. Clin Endocrinol. 1992;36:177–81.
10. Matorras R, Rodriguez F, Perez C, et al. Infertile women with and without endometriosis: A case control study of luteal phase and other infertility conditions. Acta Obstet Gynecol Scand. 1996;75:826–31.
11. Pittaway DE, Maxson W, Daniell J, et al. Luteal phase defects in infertility patients with endometriosis. Fertil Steril. 1983;39:712–3.
12. Hirschowitz JS, Soler NG, Wortsman J. The galactorrhea–endometriosis syndrome. Lancet. 1978;1:896–8.
13. Mio Y, Toda T, Harada T, Terakawa N. Luteinized unruptured follicle in the early stages of endometriosis as a cause of unexplained infertility. Am J Obstet Gynecol. 1992;167:271–3.
14. Wheeler JM, Johnston BM, Malinak LR. The relationship of endometriosis to spontaneous abortion. Fertil Steril. 1983;39:656–60.
15. Burns WN, Schenken RS. Pathophysiology of endometriosis-associated infertility. Clin Obstet Gynecol. 1999;42:586–610.
16. Thomas EJ, Lenton EA, Cooke ID. Follicle growth patterns and endocrinological abnormalities in infertile women with minor degrees of endometriosis. Br J Obstet Gynaecol. 1986;93:852–8.
17. Kusuvara K. Luteal function in infertile patients with endometriosis. Am J Obstet Gynecol. 1992;167:274–7.
18. Matalliotakis I, Panidis D, Vlassis G, et al. PRL, TSH and their response to the TRH test in patients with endometriosis before, during, and after treatment with danazol. Gynecol Obstet Invest. 1996;42:183–6.
19. Pittaway DE, Vernon C, Fayed JA. Spontaneous abortions in women with endometriosis. Fertil Steril. 1988;50:711–5.
20. Matorras R, Rodriguez F, Gutierrez de Teran G, et al. Endometriosis and spontaneous abortion rate: A cohort study in infertile women. Eur J Obstet Gynecol Reprod Biol. 1998;77:101–5.

21. Senturk LM, Arici A. Immunology of endometriosis. *J Reprod Immunol.* 1999;43:67–83.
22. De Ziegler D, Borges B, Chapron C. Endometriosis and infertility: Patho-physiology and management. *Lancet.* 2010;376:730–8.
23. Yoshida S, Harada T, Iwabe T, et al. A combination of interleukin-6 and its soluble receptor impairs sperm motility: Implications in infertility associated with endometriosis. *Hum Reprod.* 2004;19:1821–5.
24. Carli C, Leclerc P, Metz CN, Akoum A. Direct effect of macrophage migration inhibitory factor on sperm function: Possible involvement in endometriosis-associated infertility. *Fertil Steril.* 2007;88(4 Suppl):1240–7.
25. Iborra A, Palacio JR, Martinez P. Oxidative stress and autoimmune response in the infertile woman. *Chem Immunol Allergy.* 2005;88:150–62.
26. Dun EC, Taylor RN, Wieser F. Advances in the genetics of endometriosis. *Genome Med.* 2010;2:75.
27. Papari E, Noruzinia M, Kashani L, Foster WG. Identification of candidate microRNA markers of endometriosis with the use of next-generation sequencing and quantitative real-time polymerase chain reaction. *Fertil Steril.* 2020;113(6):1232–41.
28. Marí-Alexandre J, Barceló-Molina M, Belmonte-López E, et al. Micro-RNA profile and proteins in peritoneal fluid from women with endometriosis: Their relationship with sterility. *Fertil Steril.* 2018 Apr;109(4):675–84.e2.
29. Kunz G, Beil D, Huppert P, Noe M, et al. Adenomyosis in endometriosis—Prevalence and impact on fertility. Evidence from magnetic resonance imaging. *Hum Reprod.* 2005;20:2309–16.
30. Hughes E, Brown J, Collins JJ, et al. Ovulation suppression for endometriosis for women with subfertility. *Cochrane Database Syst Rev.* 2007;2007(3):CD000155. DOI: [10.1002/14651858.CD000155.pub2](https://doi.org/10.1002/14651858.CD000155.pub2).
31. Marcoux S, Maheux R, Berube S. The Canadian Collaborative Group on Endometriosis. Laparoscopic surgery in infertile women with minimal or mild endometriosis. *N Engl J Med.* 1997;336:217–22.
32. Gruppo Italiano per lo Studio dell'Endometriosi. Ablation of lesions or no treatment in minimal–mild endometriosis in infertile women: A randomized trial. *Hum Reprod.* 1999;14:1332–4.
33. Al-Inany HG, Crosignani PG, Vercellini P. Evidence may change with more trials: Concepts to be kept in mind [letters]. *Hum Reprod.* 2000;15:2447–8.
34. National Guideline Alliance (UK). Endometriosis: Diagnosis and Management. London: National Institute for Health and Care Excellence (NICE); 2017 Sep. (NICE Guideline, No. 73.) 11, Management strategies. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK536034/>.
35. Benschop L, Farquhar C, van der Poel N, Heineman MJ. Interventions for women with endometrioma prior to assisted reproductive technology. *Cochrane Database Syst Rev.* 2010;11: CD008571.
36. Hamdan M, Dunselman G, Li TC, Cheong Y. The impact of endometrioma on IVF/ICSI outcomes: A systematic review and meta-analysis. *Hum Reprod Update.* 2015;21(6):809–25.
37. Pais AS, Flagothier C, Tebache L, et al. Impact of surgical management of endometrioma on AMH levels and pregnancy rates: A review of recent literature. *J Clin Med.* 2021 Jan 22;10(3):414.
38. Alborzi S, Zahiri Sorouri Z, Askari E, et al. The success of various endometrioma treatments in infertility: A systematic review and meta-analysis of prospective studies. *Reprod Med Biol.* 2019 Jun 19;18(4):312–22.
39. Guzick DS, Carson SA, Coutifaris C, et al. Efficacy of superovulation and intrauterine insemination in the treatment of infertility. *N Engl J Med.* 1999;340:177–83.
40. Deaton JL, Gibson M, Blackmer KM, et al. A randomized, controlled trial of clomiphene citrate and intrauterine insemination in couples with unexplained infertility or surgically corrected endometriosis. *Fertil Steril.* 1990;54:1083–8.
41. Fedele L, Parazzini F, Radici E, et al. Buserelin acetate versus expectant management in the treatment of infertility associated with mild endometriosis: A randomized clinical trial. *Fertil Steril.* 1992;58:28–31.
42. Nuojua-Huttunen S, Tomas C, Bloigu R, et al. Intrauterine insemination treatment in subfertility: An analysis of factors affecting outcome. *Hum Reprod.* 1999;14:698–703.
43. Omland A, Tanbo T, Dale PO, Abyholm T. Artificial insemination by husband in unexplained infertility compared with infertility associated with peritoneal endometriosis. *Hum Reprod.* 1998;13:2602–5.
44. Hughes EG. The effectiveness of ovulation induction and intrauterine insemination in the treatment of persistent infertility: A meta-analysis. *Hum Reprod.* 1997;12:1865–72.
45. Tummon IS, Asher LJ, Martin JS, Tulandi T. Randomized controlled trial of superovulation and insemination for infertility associated with minimal or mild endometriosis. *Fertil Steril.* 1997;68:8–12.
46. Capelo F, Kumar A, Steinkampf M, Azziz R. Laparoscopic evaluation following failure to achieve pregnancy after ovulation induction with clomiphene citrate. *Fertil Steril.* 2003;80: 1450–3.
47. Werbrouck E, Spiessens C, Meuleman C, D'Hooghe T. No difference in cycle pregnancy rate and in cumulative live-birth rate between women with surgically treated minimal to mild endometriosis and women with unexplained infertility after controlled ovarian hyperstimulation and intrauterine insemination. *Fertil Steril.* 2005;86:566–71.
48. Wu HH, Wang NM, Cheng ML, Hsieh JN. A randomized comparison of ovulation induction and hormone profile between the aromatase inhibitor anastrozole and clomiphene citrate in women with infertility. *Gynecol Endocrinol.* 2007;23:76–81.
49. Abu Hashim H, El Rakawy M, Abd Elaal I. Randomized comparison of superovulation with letrozole vs. clomiphene citrate in an IUI program for women with recently surgically treated minimal to mild endometriosis. *Acta Obstet Gynecol Scand.* 2012;91: 338–45.
50. Cantineau AEP, Cohlen BJ, Klip H, et al. The addition of GnRH antagonists in intrauterine insemination cycles with mild ovarian hyperstimulation does not increase live birth rates—A randomized, doubleblinded, placebo-controlled trial. *Hum Reprod.* 2011;26:1104–11.
51. Alborzi S, Hamed B, Omidvar A, et al. A comparison of the effect of short-term aromatase inhibitor (letrozole) and GnRH agonist (triptorelin) versus case control on pregnancy rate and symptom and sign recurrence after laparoscopic treatment of endometriosis. *Arch Gynecol Obstet.* 2011 Jul;284(1):105–10.
52. The Practice Committee of the American Society of Reproductive Medicine. Endometriosis and infertility. *Fertil Steril.* 2006;86: S156–60.
53. Sanchez AM, Vanni VS, Bartiromo L, et al. Is the oocyte quality affected by endometriosis? A review of the literature. *J Ovarian Res.* 2017 Jul 12;10(1):43.
54. Kuivasaari P, Hippelainen M, Anttila M, Heinonen S. Effect of endometriosis on IVF/ICSI outcome: Stage III/IV endometriosis worsens cumulative pregnancy and live-born rates. *Hum Reprod.* 2005;20:3130–5.
55. Omland AK, Abyholm T, Fedorcsak P, et al. Pregnancy outcome after IVF and ICSI in unexplained, endometriosis-associated and tubal factor infertility. *Hum Reprod.* 2005;20:722–7.
56. Bishop LA, Gunn J, Jahandideh S, et al. Endometriosis does not impact live-birth rates in frozen embryo transfers of euploid blastocysts. *Fertil Steril.* 2021 Feb;115(2):416–22.
57. Kim SJ, Choo CW, Kim SK, et al. The effects of letrozole on women with endometriosis undergoing ovarian stimulation for in vitro fertilization. *Gynecol Endocrinol.* 2020 Mar;36(3):257–60.
58. Barnhart K, Dunsmoor-Su R, Coutifaris C. Effect of endometriosis on in vitro fertilization. *Fertil Steril.* 2002;77:1148–55.

59. Hamdan M, Omar SZ, Dunselman G, Cheong Y. Influence of endometriosis on assisted reproductive technology outcomes: A systematic review and metaanalysis. *Obstet Gynecol.* 2015;125:79–88.
60. Somigliana E, Garcia-Velasco J. Treatment of infertility associated with deep endometriosis: Definition of therapeutic balances. *Fertil Steril.* 2015;104:764–70.
61. Chilliak CF, Acosta AA, Garcia JE, et al. The role of in vitro fertilization in infertile patients with endometriosis. *Fertil Steril.* 1985;44:56–61.
62. Oehninger S, Acosta AA, Kreiner D, et al. In vitro fertilization and embryo transfer IVF/ET: An established and successful therapy for endometriosis. *J In Vitro Fert Embryo Transf.* 1988;5:249–56.
63. Matson PL, Yovich JL. The treatment of infertility associated with endometriosis by in vitro fertilization. *Fertil Steril.* 1986;46:432–4.
64. Yovich JL, Matson PL, Richardson PA, Hilliard C. Hormonal profiles and embryo quality in women with severe endometriosis treated by in vitro fertilization and embryo transfer. *Fertil Steril.* 1988;50:308–13.
65. Yovich JL, Matson PL. The influence of infertility etiology on the outcome of IVF-ET and GIFT treatments. *Int J Fertil.* 1990;35:26–33.
66. Chang MY, Chiang CH, Hsieh TT, et al. The influence of endometriosis on the success of gamete intrafallopian transfer GIFT. *J Assist Reprod Genet.* 1997;14:76–82.
67. Dlugi AM, Loy RA, Dieterle S, et al. The effect of endometriomas on in vitro fertilization outcome. *J In Vitro Fert Embryo Transf.* 1989;6:338–41.
68. Somigliana E, Infantino M, Benedetti F, et al. The presence of ovarian endometriomas is associated with a reduced responsiveness to gonadotropins. *Fertil Steril.* 2006;86:192–6.
69. Sanchez AM, Vigano P, Somigliana E, et al. The distinguishing cellular and molecular features of the endometriotic ovarian cyst: From pathophysiology to the potential endometrioma-mediated damage to the ovary. *Hum Reprod Update.* 2014;20:217–30.
70. Orazov MR, Radzinsky VY, Ivanov II, et al. Oocyte quality in women with infertility associated endometriosis. *Gynecol Endocrinol.* 2019;35(sup1):24–6.
71. Dmowski WP, Rana N, Michalowska J, et al. The effect of endometriosis, its stage and activity, and of autoantibodies on in vitro fertilization and embryo transfer success rates. *Fertil Steril.* 1995;63:555–62.
72. Bergendal A, Naffah S, Nagy C, et al. Outcome of IVF in patients with endometriosis in comparison with tubal factor infertility. *J Assist Reprod Genet.* 1998;15:530–4.
73. Geber S, Paraschos T, Atkinson G, et al. Results of IVF in patients with endometriosis: The severity of the disease does not affect outcome, or the incidence of miscarriage. *Hum Reprod.* 1995;10:1507–11.
74. Inoue M, Kobayashi Y, Honda I, et al. The impact of endometriosis on the reproductive outcome of infertile patients. *Am J Obstet Gynecol.* 1992;167:278–82.
75. Pal L, Shifren JL, Isaacson KB, et al. Impact of varying stages of endometriosis on the outcome of in vitro fertilization–embryo transfer. *J Assist Reprod Genet.* 1998;15:27–31.
76. Olivennes F, Feldberg D, Liu HC, et al. Endometriosis: A stage by stage analysis—The role of in vitro fertilization. *Fertil Steril.* 1995;64:392–8.
77. Oehninger S, Brzyski RG, Muasher SJ, et al. In vitro fertilization and embryo transfer in patients with endometriosis: Impact of a gonadotrophin releasing hormone agonist. *Hum Reprod.* 1989;4:541–4.
78. Dicker D, Goldman JA, Levy T, et al. The impact of long-term gonadotropin-releasing hormone analogue treatment on preclinical abortions in patients with severe endometriosis undergoing in vitro fertilization–embryo transfer. *Fertil Steril.* 1992;57:597–600.
79. Rickes D, Nickel I, Kropf S, Kleinstein J. Increased pregnancy rates after ultralong postoperative therapy with gonadotropin-releasing hormone analogs in patients with endometriosis. *Fertil Steril.* 2002;78:757–62.
80. Chedid S, Camus M, Smitz J, et al. Comparison among different ovarian stimulation regimens for assisted procreation procedures in patients with endometriosis. *Hum Reprod.* 1995;10:2406–11.
81. Nakamura K, Oosawa M, Kondou I, et al. Metrodin stimulation after prolonged gonadotropin releasing hormone agonist pretreatment for in vitro fertilization in patients with endometriosis. *J Assist Reprod Genet.* 1992;9:113–7.
82. Marcus SF, Edwards RG. High rates of pregnancy after long-term down-regulation of women with severe endometriosis. *Am J Obstet Gynecol.* 1994;171:812–7.
83. Mahadevan MM, Trounson AO, Leeton JF. The relationship of tubal blockage, infertility of unknown cause, suspected male infertility, and endometriosis to the success of in vitro fertilization and embryo transfer. *Fertil Steril.* 1983;40:755–62.
84. Wardle PG, Mitchell JD, McLaughlin EA, et al. Endometriosis and ovulatory disorder: Reduced fertilisation in vitro compared with tubal and unexplained infertility. *Lancet.* 1985;2:236–9.
85. Sharma V, Riddle A, Mason BA, et al. An analysis of factors influencing the establishment of a clinical pregnancy in an ultrasound-based ambulatory in vitro fertilization program. *Fertil Steril.* 1988;49:468–78.
86. Arici A, Oral E, Bukulmez O, et al. The effect of endometriosis on implantation: Results from the yale university in vitro fertilization and embryo transfer program. *Fertil Steril.* 1996;65:603–7.
87. Surrey ES, Silverberg KM, Surrey MW, Schoolcraft WB. Effect of prolonged gonadotropin-releasing hormone agonist therapy on the outcome of in vitro fertilization–embryo transfer in patients with endometriosis. *Fertil Steril.* 2002;78:699–704.
88. Kaponis A, Chatzopoulos G, Paschopoulos M, et al. Ultralong administration of gonadotropin-releasing hormone agonists before in vitro fertilization improves fertilization rate but not clinical pregnancy rate in women with mild endometriosis: A prospective, randomized, controlled trial. *Fertil Steril.* 2020 Apr;113(4):828–35.
89. Surrey ES. To suppress or not to suppress? If that is the question, has it been answered? *Fertil Steril.* 2020;113(4):763–4.
90. Surrey ES. Endometriosis and assisted reproductive technologies: Maximizing outcomes. *Semin Reprod Med.* 2013;31(2):154–63.
91. de Ziegler D, Gayet V, Aubriot PF, et al. Use of oral contraceptives in women with endometriosis before assisted reproduction treatment improves outcomes. *Fertil Steril.* 2010;94:2796–9.
92. Tomassetti C, Beukeleirs T, Conforti A, et al. The ultra-long study: A randomized controlled trial evaluating long-term GnRH down-regulation prior to ART in women with endometriosis. *Hum Reprod.* 2021 Sep 18;36(10):2676–86.
93. Guo H, Li J, Shen X, et al. Efficacy of different progestins in women with advanced endometriosis undergoing controlled ovarian hyperstimulation for in vitro fertilization—a single-center non-inferiority randomized controlled trial. *Front Endocrinol (Lausanne).* 2020 Mar 20;11:129.
94. Tamura H, Yoshida H, Kikuchi H, et al. The clinical outcome of dienogest treatment followed by in vitro fertilization and embryo transfer in infertile women with endometriosis. *J Ovarian Res.* 2019 Dec 12;12(1):123.
95. Recai P, Onalan G, Kaya C. GnRH agonist and antagonist protocols for stage I-II endometriosis and endometrioma in in vitro fertilization/intracytoplasmic sperm injection cycles. *Fertil Steril.* 2007;88:832–8.
96. Yanushpolsky EH, Best CL, Jackson KV, et al. Effects of endometriomas on oocyte quality, embryo quality, and pregnancy rates in in vitro fertilization cycles: A prospective, case–controlled study. *J Assist Reprod Genet.* 1998;15:193–7.

97. Pellicer A, Oliveira N, Ruiz A, et al. Exploring the mechanisms of endometriosis-related infertility: An analysis of embryo development and implantation in assisted reproduction. *Hum Reprod*. 1995; 10(Suppl 2): 91–7.
98. Tanbo T, Omland A, Dale PO, Abyholm T. Transfer in vitro fertilization/embryo transfer in unexplained infertility and minimal peritoneal endometriosis. *Acta Obstet Gynecol Scand*. 1995;74:539–43.
99. Brizek CL, Schlaff S, Pellegrini VA, et al. Increased incidence of aberrant morphological phenotypes in human embryogenesis—An association with endometriosis. *J Assist Reprod Genet*. 1995;12:106–12.
100. Suzuki T, Izumi SI, Matsubayashi H, et al. Impact of ovarian endometrioma on oocyte and pregnancy outcome in in vitro fertilization. *Fertil Steril*. 2005;83:908–13.
101. Komsky-Elbaz A, Raziel A, Friedler S, et al. Conventional IVF versus ICSI in sibling oocytes from couples with endometriosis and normozoospermic semen. *J Assist Reprod Genet*. 2013 Feb;30(2):251–7.
102. Shebl O, Sifferlinger I, Habelsberger A, et al. Oocyte competence in in vitro fertilization and intracytoplasmic sperm injection patients suffering from endometriosis and its possible association with subsequent treatment outcome: A matched case-control study. *Acta Obstet Gynecol Scand*. 2017 Jun;96(6):736–44.
103. Vaiarelli A, Venturella R, Cimadomo D, et al. Endometriosis shows no impact on the euploid blastocyst rate per cohort of inseminated metaphase-II oocytes: A case-control study. *Eur J Obstet Gynecol Reprod Biol*. 2021;256:205–10.
104. Niu Z, Chen Q, Sun Y, Feng Y. Long-term pituitary downregulation before frozen embryo transfer could improve pregnancy outcomes in women with adenomyosis. *Gynecol Endocrinol*. 2013;29:1026–30.
105. Sallam HN, Garcia-Velasco JA, Dias S, Arici A. Long-term pituitary down-regulation before in vitro fertilization (IVF) for women with endometriosis. *Cochrane Database Syst Rev*. 2006;1:CD004635.
106. Simsek Y, Celik O, Karaer A, et al. Therapeutic efficiency of atosiban, an oxytocin receptor blocking agent in the treatment of experimental endometriosis. *Arch Gynecol Obstet*. 2012 Sep;286(3): 777–83.
107. Gunther R, Walker C. Adenomyosis. [Updated 2021 Jul 22]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021.
108. Sacco K, Portelli M, Pollacco J, et al. The role of prostaglandin E2 in endometriosis. *Gynecol Endocrinol*. 2012 Feb;28(2):134–8.
109. He Y, Wu H, He X, et al. Administration of atosiban in patients with endometriosis undergoing frozen-thawed embryo transfer: A prospective, randomized study. *Fertil Steril*. 2016 Aug;106(2): 416–22.
110. Simon C, Gutierrez A, Vidal A, et al. Outcome of patients with endometriosis in assisted reproduction: Results from in vitro fertilization and oocyte donation. *Hum Reprod*. 1994;9:725–9.
111. Sung L, Mukherjee T, Takeshige T, et al. Endometriosis is not detrimental to embryo implantation in oocyte recipients. *J Assist Reprod Genet*. 1997;14:152–6.
112. Diaz I, Navarro J, Blasco L, et al. Impact of stage III–IV endometriosis on recipients of sibling oocytes: Matched case-control study. *Fertil Steril*. 2000;74:31–4.
113. Isaacs JD Jr, Hines RS, Sopelak VM, Cowan BD. Ovarian endometriomas do not adversely affect pregnancy success following treatment with in vitro fertilization. *J Assist Reprod Genet*. 1997;14:551–3.
114. Stephansson O, Kieler H, Granath F, Falconer H. Endometriosis, assisted reproduction technology, and risk of adverse pregnancy outcome. *Hum Reprod*. 2009;24:2341–7.
115. Fernando S, Breheny VS, Jaques AM, et al. Preterm birth, ovarian endometrioma, and assisted reproduction technologies. *Fertil Steril*. 2009;91:325–30.
116. Kobayashi H, Kawahara N, Ogawa K, Yoshimoto C. A relationship between endometriosis and obstetric complications. *Reprod Sci*. 2020;27(3):771–8.
117. Farland LV, Prescott J, Sasamoto N, et al. Endometriosis and risk of adverse pregnancy outcomes. *Obstet Gynecol*. 2019 Sep;134(3):527–36.
118. Pagidas K, Falcone T, Hemmings R, Miron P. Comparison of reoperation for moderate stage III and severe stage IV endometriosis-related infertility with in vitro fertilization–embryo transfer. *Fertil Steril*. 1996;65:791–5.
119. Jacobson T, Barlow D, Koninckx P, et al. Laparoscopic surgery for subfertility associated with endometriosis. *Cochrane Database Syst Rev*. 2002;4:CD001398.
120. Garcia-Velasco JA, Mahutte NG, Corona J, et al. Removal of endometriomas before in vitro fertilization does not improve fertility outcomes: A matched, case-control study. *Fertil Steril*. 2004;81:1194–7.
121. Tsoumpou I, Kyrgiou M, Gelbaya TA, Nardo LG. The effect of surgical treatment for endometrioma on in vitro fertilization outcomes: A systematic review and meta-analysis. *Fertil Steril*. 2009;92:75–87.
122. Mingué Y, Rubio C, Bernal A, et al. The impact of endometriosis in couples undergoing intracytoplasmic sperm injection because of male infertility. *Hum Reprod*. 1997;12:2282–5.
123. Bianchi PH, Pereira RM, Zanatta A, et al. Extensive excision of deep infiltrative endometriosis before in vitro fertilization significantly improves pregnancy rates. *J Minim Invasive Gynecol*. 2009;16:174–80.
124. Littman E, Giudice L, Lathi R, et al. Role of laparoscopic treatment of endometriosis in patients with failed in vitro fertilization cycles. *Fertil Steril*. 2005;84:1574–8.
125. Nada AM, El-Noury A, Al-Inany H, et al. Effect of laser-assisted zona thinning, during assisted reproduction, on pregnancy outcome in women with endometriosis: Randomized controlled trial. *Arch Gynecol Obstet*. 2018 Feb;297(2):521–8.
126. Stochino-Loi E, Major AL, Gillon TER, et al. Metformin, the rise of a new medical therapy for endometriosis? A systematic review of the literature. *Front Med (Lausanne)*. 2021 May 11;8:581311.
127. Kimber-Trojnar Ž, Dłuski DF, Wierzchowska-Opoka M, et al. Metformin as a potential treatment option for endometriosis. *Cancers (Basel)*. 2022 Jan 24;14(3):577.
128. Sansone AM, Brooke VH, Young RB, et al. “Evaluation of BCL6 and SIRT1 as non-invasive diagnostic markers of endometriosis” *Curr Issues Mol Biol*. 2021;43(3):1350–60.
129. Soto E, Luu TH, Liu X, et al. Laparoscopy vs. robotic surgery for endometriosis (LAROSE): A multicenter, randomized, controlled trial. *Fertil Steril*. 2017 Apr;107(4):996–1002.

POLYCYSTIC OVARY SYNDROME AND ASSISTED REPRODUCTION

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Introduction

Poly cystic ovary syndrome (PCOS) is a common condition affecting approximately 10%–15% of women. PCOS comprises a heterogeneous collection of signs and symptoms that gather together to form a spectrum of a disorder with a mild presentation in some women and a severe disturbance of reproductive, endocrine, and metabolic function in others. The pathophysiology of the PCOS appears to be multifactorial with a plethora of pathophysiological and genetic origins that lead to the heterogeneity of expression of the syndrome. There are significant ethnic variations in the manifestation of PCOS which are also influenced by environmental factors ranging from maternal health and in utero growth to lifestyle, body weight, and metabolic health.

The definition of PCOS has been much debated. Key features include menstrual cycle disturbance, hyperandrogenism, and obesity. There are many extra-ovarian aspects to the pathophysiology of PCOS, yet ovarian dysfunction is central. Terminology is important, and there has been an appropriate shift away from the term *polycystic ovarian disease* to the more commonly accepted *poly cystic ovary syndrome*. Furthermore, the term Stein-Leventhal syndrome disappeared some 30 years ago, so named after the first to describe the condition in modern times [1].

In vitro fertilization (IVF) should be viewed as third-line treatment for those with PCOS [2]. In the absence of known causative factors of infertility such as tubal or sperm abnormalities, a methodical approach to treatment should first include lifestyle modification and an efficacious trial of ovulation induction therapies. For those who remain refractory to different regimens of ovulation induction (OI), the move to IVF with the associated risk of ovarian hyperstimulation syndrome (OHSS) becomes justified. We recommend at least six, if not nine, cycles of OI with confirmed ovulation before moving on to IVF. This will, of course, depend upon the age of the woman and other reproductive factors. If ovulation is not achieved with first-line therapy, such as letrozole, then gonadotropin therapy is indicated before IVF [2]. The presence of polycystic ovaries, which may exist in the absence of symptoms of the full-blown syndrome, is also a major risk factor for OHSS, necessitating careful planning of gonadotrophin stimulation.

Definition

Until 20 years ago there was no international consensus either on the definition of the syndrome or, indeed, on what constitutes a polycystic ovary. At a joint European Society of Human Reproduction and Embryology (ESHRE)/American Society for Reproductive Medicine (ASRM) consensus meeting in Rotterdam in 2003, we agreed to a pragmatic and all-encompassing definition of the PCOS, namely, the presence of two out of the following three criteria: (1) oligo-ovulation and/or anovulation; (2) hyperandrogenism (clinical and/or biochemical); and (3) polycystic ovaries, with the exclusion of other aetiologies [3]. These

“Rotterdam criteria” have since been debated by other bodies but nonetheless have gained widespread acceptance and have been agreed to by both the WHO and the Global PCOS Alliance in the latest international guidelines [3, 4]. In 2003, the morphology of the polycystic ovary also was redefined as an ovary with 12 or more follicles measuring 2–9 mm in diameter and/or increased ovarian volume ($>10 \text{ cm}^3$) [5]. Again, this definition has been refined further to include the presence of at least 20 follicles [4].

Prevalence and spectrum of PCOS

Polycystic ovaries (PCO) are commonly detected by pelvic ultrasound, with estimates of the prevalence of PCO in the general population being in the order of 20%–33% [6–8]. However, not all women with polycystic ovaries demonstrate the clinical and biochemical features that define PCOS. These features include menstrual cycle disturbances (oligomenorrhoea and amenorrhoea), signs of androgen excess/hyperandrogenism (hirsutism, acne, and alopecia); and abnormalities of biochemical profiles including elevated serum concentrations of luteinizing hormone (LH), testosterone (T), androstenedione, and anti-Müllerian hormone (AMH). Obesity and hyperinsulinaemia are associated features, although only 40%–50% of women with PCOS are overweight. Presentation of the syndrome is so varied that one, all, or any combination of the aforementioned features may be present in association with an ultrasound picture of polycystic ovaries [9].

There is considerable heterogeneity of symptoms and signs among women with PCOS, and for an individual, these symptoms and signs may change over time. PCOS is the commonest cause of anovulatory infertility and there are significant challenges in providing effective and safe ovulation induction therapy [2]. Although, paradoxically, whilst women with PCOS may take longer to conceive, they end up with similar family sizes to the general population, their menstrual cycles tend to become more regular towards the end of their reproductive years, and their ovaries are fertile for longer. For older women, there are significant associations with metabolic problems, diabetes, and cardiovascular disease, although these do not always translate to higher mortality. PCOS is often familial and various aspects of the syndrome may be differentially inherited. There are several interlinking factors that affect expression of PCOS [10]. A gain in weight is associated with a worsening of symptoms, whereas weight loss ameliorates the endocrine and metabolic profile and symptomatology [11]. Although weight reduction does not necessarily normalize the situation, it certainly improves response to therapy, whether for fertility or other aspects of the syndrome.

Aetiology and pathophysiology

The aetiology of PCOS has proved difficult to elucidate with up to 10 phenotypes and ethnic variations in the expression of androgen excess, ovarian function, and insulin resistance [12]. It is thought that the genetic propensity to gain weight easily

in some populations may historically have preserved fertility in times of famine (the “thrifty genotype”), as underweight women do not have the nutrition to sustain a healthy pregnancy and so the gene(s) that lead to PCOS may have conferred an evolutionary advantage [13].

There is strong evidence that PCOS runs in families and also that male relatives may also have an increased risk of insulin resistance. It appears that approximately 50% of first-degree relatives—that is sisters, mothers, or daughters—of women with PCOS are likely to have the syndrome too. There have been numerous attempts to elucidate the genes that may be involved, with the latest genome-wide association studies (GWAS) identifying several potential loci of interest (in particular *THADA*, *FSHR*, *INS-VNTR*, and *DENND1A*), but still no firm conclusions [14, 15]. Interestingly, a paternal history of diabetes mellitus has a greater influence than maternal diabetes mellitus on the risk of the daughter developing PCOS [16]. Recent data that DNA methylation and other epigenetic changes play a role in the development of PCOS [17] and the recent evidence that sperm methylation is affected by male obesity [18] and that this can influence the methylation status of genes for the offspring [19] combine to provide an intriguing hypothesis on paternal influences on the development of PCOS. There also appears to be a higher incidence of insulin resistance and dyslipidaemia in men with unexplained oligozoospermia, suggesting a link between insulin resistance and spermatogenesis via insulin-like growth factor (IGF-1) [20].

Whilst the manifestations of PCOS are well characterized, the pathophysiology of the ovarian dysfunction originates in a number of ways. The ovarian dysfunction is evidenced by numerous immature egg-containing follicles (the eponymous “cysts”) that fail to grow and ovulate in a coordinated fashion because of the hyper-secretion of luteinizing hormone and insulin promoting overproduction of androgens and excess ovarian anti-Müllerian hormone (AMH) production disrupting follicle recruitment [21]. The scene is thought to be set in utero through the combination of the maternal endocrine milieu and placental function influencing fetal hypothalamic function, gonadal development, and fat deposition. Some of these elements may be inherited through genetic variations or further affected by epigenetic factors and the interaction of maternal and in utero environments.

There is an expanding literature on transgenerational medicine in relation to PCOS. It has been shown in a Swedish nationwide register that there is a fivefold increased risk of PCOS in daughters of mothers with PCOS [22], and in a Chilean case-control study, 71% of the daughters of women with PCOS were found themselves to have PCOS [22]. These authors have used a mouse model to explore how *in utero* exposures might influence the development of the PCOS phenotype through a series of inter- and transgenerational studies, designed to separate the influence of maternal obesity and prenatal androgen exposure [22]. Other animal models used to study PCOS include sheep [23] and non-human primates [24]. Whilst it is possible to administer androgens prenatally to provoke the development of a PCOS-like syndrome with respect to ovarian morphology, hyperandrogenism, adipocyte function, and metabolic disturbance, these models will only ever be approximate surrogates for the complexities observed in humans. Mouse models have also shown that prenatal administration of AMH may lead to hyperactivated gonadotropin-releasing hormone neurons in the hypothalamus and consequent hyperandrogenism, suggesting that there is more at play than simply the delivery of exogenous androgens [25]. This

all provides an intriguing glimpse of a potential mechanism for transmission of the effects of maternal androgen and obesity environments on future generations. The fertile capacity of oocytes is acquired gradually during oogenesis and is dependent on the correct genetic, epigenetic, and metabolic programming of the germ line through the individual’s life course, as well as the gradual and timely acquisition of the payload of RNA, proteins, and key cytoplasmic organelles such as the mitochondria. The preparation of oocytes for fertilization involves meiotic division to deliver half the chromosomal complement to the embryo as well as cytoplasmic maturation events that protect and repackaging key maternal RNAs for later use by the embryo. Importantly, the maternal and paternal genomes do not contribute equally to embryo fate such that cytoplasmic inventory of an early embryo is inherited entirely from the parent oocyte [26, 27]. Similarly, the fertilization and developmental competence of the embryo are dependent on efficient metabolism driven by mitochondria that are derived also exclusively from the oocyte [28]. Disruption of these vital components of oocyte biology will reduce fertility and significantly influence embryo viability and implantation potential. This biological insight aids interpretation of how in utero exposure to androgen excess and obesity, such as occurs during PCOS, may affect RNA binding and gene expression and disrupt the mitochondria and metabolic machinery at a critical time that directly impacts on oocyte quality and compromises the fertility and metabolic health of future generations [27].

Reproductive health in PCOS

In addition to anovulation, there may be other factors that contribute to subfertility in women with PCOS, including the effects of obesity and metabolic, inflammatory, and endocrine abnormalities on oocyte quality and fetal development. Oocytes from PCOS may exhibit reduced developmental competence, with a reduced ability to complete meiosis, achieve fertilization, and develop into a normal embryo. Ovarian hyperandrogenism and hyperinsulinemia may promote premature granulosa cell luteinization; furthermore, paracrine dysregulation of growth factors may disrupt the intrafollicular environment, alter granulosa cell–oocyte interactions, and impair cytoplasmic and/or nuclear maturation of oocytes [29]. There is variability, however, and oocyte quality, fertilization, and implantation rates in women with PCOS may be normal [30].

PCOS is associated with metabolic disturbances that include impaired insulin signalling and glucose metabolism in ovarian follicles [31]. It is likely that the metabolic lesion in the follicle precipitates an altered metabolic milieu throughout oogenesis, which may have downstream consequences for oocyte energy generation. This may lead to reduced expression of genes encoding oxidative phosphorylation [32]. Altered expression of key genes associated with chromosome alignment and segregation has also been attributed to hyperandrogenaemia [33]. Indeed, it has been shown that differences in metabolism exist in oocytes derived from women with PCOS, and this is associated with chromosomal pre-division; that is, premature separation of sister chromatids [34]. During early pregnancy, the embryo may be exposed to androgen excess in utero, which may have long-term effects, particularly on female offspring. Fetal hyperandrogenism may disturb epigenetic programming, particularly those genes regulating reproduction and metabolism [35, 36]. It is also possible that transgenerational effects may be related to the potential influences of hyperinsulinemia and its effect on the intrauterine environment.

In a meta-analysis in which pregnancy outcomes in women with PCOS were compared with controls, women with PCOS demonstrated a significantly higher risk of developing gestational diabetes mellitus (GDM; odds ratio [OR] 2.94; 95% confidence interval [CI]: 1.70–5.08), pregnancy-induced hypertension (OR 3.67; 95% CI: 1.98–6.81), pre-eclampsia (OR 3.47; 95% CI: 1.95–6.17), and preterm birth (OR 1.75; 95% CI: 1.16–2.62). Their babies had a significantly higher risk of admission to a neonatal intensive care unit (OR 2.31; 95% CI: 1.25–4.26) and a higher perinatal mortality (OR 3.07; 95% CI: 1.03–9.21), unrelated to multiple births [37]. In addition, GDM may also result in fetal macrosomia. Obesity in its own right is associated with several adverse pregnancy outcomes, including spontaneous miscarriage, pre-eclampsia, GDM, congenital anomalies (e.g. cardiac and spina bifida), and fetal macrosomia [38].

Biochemistry within the polycystic ovary

Hyperandrogenism (HA), in conjunction with hyperinsulinemia, is a fundamental feature of PCOS. The hormonal interplay between key hormones in PCOS adds to perturbed folliculogenesis and oocyte competence [39]. Hyperandrogenism influences the hypothalamic–pituitary–ovarian axis in a number of ways. Furthermore, there are additional mechanisms that lead to increased GnRH pulsatility, hypersecretion of LH, premature granulosa cell luteinization, and abnormal oocyte maturation. Through direct and indirect mechanisms, HA impairs oocyte competence, including causing premature oocyte maturation and activated pro-apoptotic signalling pathways in oocytes [40]. It has been shown that follicular testosterone levels are significantly elevated in PCOS, especially in follicles with meiotically incompetent oocytes, thereby contributing to the reduced fertilization rates seen in PCOS [41].

Anti-Müllerian hormone (AMH) is elevated in women with PCOS. High levels of AMH exacerbate follicular FSH resistance, through inhibition of aromatase activity. Failed conversion of androgen to oestrogen leads to chronic HA, interrupting the follicles' ability to undergo cyclic recruitment among selectable follicles [33].

Oxidative stress is caused by an imbalance between pro-oxidant molecules and antioxidants, culminating in increased concentrations of reactive oxygen species (ROS). Low levels of ROS in follicular fluid promote oocyte maturation and contribute to the release of a competent oocyte at the time of ovulation. Elevated ROS levels induce DNA damage and can interfere with oocyte competence. A hyperandrogenic ovarian milieu promotes oxidative stress, interfering with oxygen attainment and oxidative metabolism required for oocyte development [39].

Both *in vivo* and *in vitro* data confirm that the theca cells of PCOS patients have a generalized overactive steroidogenesis. PCOS patients have a tendency to an excess of oestradiol at all stages of follicular maturation. This excess steroidogenesis is partly due to availability of excess androgen substrate for aromatase activity, as well as an excessive response of follicle development and oestradiol secretion to FSH. Serum androgen levels rise during ovarian stimulation and are higher in women with PCOS. Increased levels are thought to negatively impact on pregnancy outcome. Markers of endometrial receptivity include glycodeolin, a secretory protein in the endometrium. A positive correlation exists between successful conception cycles following IVF and glycodeolin. Increased androgen levels have been found to reduce glycodeolin in women with PCOS and recurrent miscarriage [42]. Hyperandrogenaemia has been shown to contribute to

altered gene expression associated with chromosome alignment and segregation [33]. Furthermore, there is altered metabolism in oocytes from women with PCOS leading to premature separation of sister chromatids. Exposure to excess androgens in utero can have far-reaching effect, including epigenetic programming, particularly on genes regulating reproduction and metabolism [44].

LH excess is a cause of ovarian hyperandrogenism of PCOS, in view of the stimulatory effect of LH on theca cells, and hypersecretion of LH is predominantly a feature of women with PCOS who are slim. Those 60% of women who have normal LH levels, are usually overweight and instead insulin and IGF-1 promote androgen excess. Furthermore, the theca cells of PCOS women hyper-respond to gonadotropins and produce excess androgens due to an escape of their normal downregulation to gonadotropins, thereby linking this dysregulation to excess of insulin and IGF-1. Indeed, insulin acts as a co-gonadotropin and also amplifies the effects of T by suppressing SHBG.

Inhibin is an FSH-inducible factor, produced by the ovaries, that is capable of interfering with the downregulation of steroidogenesis. Plasma inhibin and androstenedione concentrations correlate, and women with PCOS have elevated serum inhibin B [45]. This finding helps to explain the relatively low serum concentrations of FSH compared with LH in anovulatory women with PCOS. Because inhibin stimulates androgen production, and androgens, in turn, stimulate inhibin secretion, there is a potential for the development of a vicious cycle within the ovary that would inhibit follicle development. LH also acts on granulosa cells in the presence of insulin, thereby leading to premature luteinization, maturational arrest, and excess androgen production [46]. In summary, as a consequence of dysregulation of androgen synthesis within the ovary, women with PCOS have ovarian hyperresponsiveness to gonadotropins: that of thecal cells to LH explaining the excess androgens, and that of granulosa cells to FSH leading to increased oestrogens.

Metabolic health and obesity in PCOS

There is a large body of evidence now on the incidence of metabolic complications of pregnancy in women with PCOS, with a recent large data set including almost 15,000 women (PCOS $n = 14882$; "normal" $n = 9081906$) [47]. At baseline, more pregnant women with PCOS were obese (22.3% vs 3.5%, $P < 0.001$), had pre-gestational diabetes (4.1% vs 0.9%, $P < 0.001$), chronic hypertension (8.4% vs 1.8%, $P < 0.001$), and had treated thyroid disease (12.6% vs 2.4%, $P < 0.001$)—the latter perhaps an unexpected association. Women with PCOS were more likely to have undergone IVF treatment (2.4% vs 0.1%, $P < 0.001$) and have multiple pregnancies (5.9% vs 1.5%, $P < 0.001$). In all pregnancies, women with PCOS were more likely to develop gestational diabetes (adjusted odds ratio (aOR) 2.19, 95% CI 2.02–2.37), pregnancy associated hypertension (aOR 1.38, 95% CI 1.27–1.50, $P < 0.001$) and pre-eclampsia (aOR 1.29, 95% CI 1.14–1.45) [47]. This is in agreement with an earlier study which also found an increased risk of preterm birth (OR 1.75; 95% CI 1.16–2.62), an increased risk of admission of babies to neonatal intensive care (OR 2.31; 95% CI 1.25–4.26), and a higher perinatal mortality (OR 3.07; 95% CI 1.03–9.21), unrelated to multiple birth [37].

Lifestyle modification and health optimization prior to treatment and pregnancy is of paramount importance. A recent Cochrane review addressed lifestyle changes in women with PCOS and concluded that intervention may improve endocrine profile, reproductive outcome, and body mass index, although no studies assessed live birth or miscarriage rates [49]. Long-term

health optimization is key for women with PCOS, both for reproductive health and long-term overall health. The approach should ideally be long-term and sustained, as quick “fix” weight-loss programmes prior to commencing ART are unlikely to substantially alter the ability to achieve a successful pregnancy through IVF [50].

Obesity is a major factor that influences all outcomes for women with PCOS. Between 38% and 66% of women with PCOS are overweight or obese, with body mass index (BMI) correlating with the severity of phenotypic features. Clinical pregnancy rates are significantly lower in the obese in either natural or ART cycles. This reduction in pregnancy rate is seen in both fresh and frozen transfer cycles. Women with PCOS undergoing IVF who have a very high BMI of greater than 40 kg/m^2 have been shown to have a significantly reduced clinical pregnancy rate (32% vs 72%, relative risk [RR] 0.44) [51]. Cycles were further complicated by increased gonadotrophin requirement, difficult oocyte retrievals, fewer oocytes retrieved, and impaired fertilization. Embryo quality was reduced with a greater degree of embryo fragmentation. Similar findings are seen in freeze-all cycles, with a reduction in LBR (aRR = 0.66; 95% CI 0.48–0.92) and increased miscarriage rate (aRR = 1.68; 95% CI 1.01–3.09) in those with a BMI greater than 30 kg/m^2 undergoing a frozen cycle [52]. This implies that obesity influences oocyte quality perhaps more than endometrial receptivity, both of which are considered key factors in obese patients with PCOS. If weight loss can be achieved, then one could logically expect an improved outcome. A large Swedish study of obese infertile women compared a strict calorie-controlled diet for 12 weeks and a period of weight stabilization prior to IVF with those who went straight to IVF [53]. Whilst there was an improved chance of natural conception in those who achieved weight loss prior to IVF, in a subgroup analysis of those with PCOS who reduced weight by either 5 BMI points or to a BMI of less than 25, there was no difference in LBR following IVF. Therefore, short-term weight reduction may not always rectify the outcome for those who are overweight, and it may well be more important to focus on long-term lifestyle modification in order to alter the disordered hormonal and metabolic environment within which the oocyte develops and matures.

Conception alone should not be the only focus for those who are overweight. Maternal health is paramount both to improve the long-term outcome for the baby and also reduce any risks during pregnancy. In the most recent triennial report (2015–2017) into maternal death, more than 34% of women who died were obese and a further 24% were overweight [54]. Both obesity and PCOS increase the risk of developing gestational diabetes, pre-eclampsia, and preterm birth. An increased need for operative delivery predisposes to wound infection and thromboembolism. Preconception health advice and support is essential in order to reduce the spiralling effects of the obesity epidemic on fertility and childbirth.

Superovulation strategies for women with polycystic ovaries and PCOS undergoing IVF

GnRH antagonist versus agonist protocols

When considering the best protocol for superovulation in women with polycystic ovaries undergoing IVF, the aim is to maximize synchronous follicular growth with oocyte maturation. Historically, the gonadotrophin-releasing hormone (GnRH) agonist protocol appeared to provide significant benefit for women

with PCOS [55], although with improved oocyte yield and pregnancy rate came a sixfold increased incidence of OHSS. This was due in part to the longer duration and total dose of gonadotrophin required for ovarian stimulation [56]. This fact is particularly pertinent for those with PCOS, who can have an unpredictable response and then an exuberant development of follicles when the effects of pituitary suppression are overcome. The agonist may interfere with the follicle selection process, preventing atresia of small antral follicles and allowing more mid-sized follicles to develop. Furthermore, following downregulation, the low-basal levels of FSH may be sufficient to support the growth of multiple small follicles. Indeed, OHSS is usually associated with a large number of small to moderate sized follicles (<14 mm) rather than with larger, more mature follicles.

The use of GnRH agonists has now been superseded by the GnRH antagonist protocols with equivalent live birth rates but significantly reduced risks of OHSS. The most recent Cochrane review has found no difference in live birth rate between the antagonist or the long GnRH agonist protocol (OR 1.02, 95% CI 0.85–1.23; 12 RCTs, n = 2303, I² = 27%; moderate quality evidence) [57]. There is a significant reduction in the incidence of any grade of OHSS (OR 0.61, 95% CI 0.51–0.72; 36 RCTs, n = 7944, I² = 31%; moderate quality evidence) and a reduction in cycle cancellation for over-response (OR 0.47, 95% CI 0.32–0.69; 19 RCTs, n = 4256). It should be recognized that whilst over response is reduced, there may be increased cycle cancellation for poor response with the antagonist cycle (OR 1.32, 95% CI 1.06–1.65; 25 RCTs, n = 5230, I² = 68%; moderate quality evidence). There is no difference in miscarriage rate between agonist or antagonist cycles (OR 1.03, 95% CI 0.82–1.29; 34 RCTs, n = 7082, I² = 0%; moderate quality evidence) [57].

Pre-treatment strategies such as using the combined oral contraceptive pill (COCP) are often employed to time cycles for planning a clinic's workload. In high-responding women with PCOS, this may be detrimental, as some studies have shown an increased duration of stimulation and a reduction in pregnancy rate. The most recent Cochrane review for COCP use concludes that there is insufficient evidence regarding OHSS with or without the pill, but a significant reduction in pregnancy rates in the antagonist cycle (OR 0.74, 95% CI 0.58–0.95; 6 RCTs; 1335 women; I² = 0%; moderate quality evidence) when the pill is used [58].

Gonadotrophin selection

Follicle stimulating hormone exists in a number of different isoforms, dependent on the number of branching carbohydrate moieties found on the molecule. Within the menstrual cycle, the more acidic isoform predominates the early follicular cycle with a switch to the less-acidic form around ovulation. This switch is controlled by oestradiol levels. *In vivo* acidic isoforms have a longer half-life with a more controlled steroidogenic response and selective follicle growth. Less-acidic isoforms induce exponential growth, in a less selective manner, which theoretically could exacerbate OHSS with rapidly rising oestradiol levels. Although there has been interest in the ratio of FSH isoforms between gonadotrophins, in practice no difference in clinical outcome has been shown between preparations. There is no difference in outcome between recombinant or urinary-derived gonadotrophins, with respect to live birth rate (28 trials, 7339 couples, odds ratio (OR) 0.97, 95% CI 0.87–1.08) or incidence of OHSS in all women undergoing IVF (32 trials, 7740 couples, OR 1.18, 95% CI 0.86–1.61) [59]. With respect to those with PCOS, a more recent meta-analysis again confirms no difference in clinical outcome

between the types of gonadotrophin used [60]. The authors of both meta-analyses suggest that the gonadotrophin used should be selected based on cost and convenience for the individual.

Insulin resistance and metformin in the context of IVF for women with PCOS

As hyperinsulinaemia is well recognized in women with PCOS, a reasonable assumption would be that insulin-sensitizing drugs should improve many aspects of the syndrome, including reproductive outcome. Metformin, an oral biguanide, is the most widely researched agent in this category. Metformin reduces hepatic gluconeogenesis, increases peripheral utilization of glucose, and mediates receptor kinase activity in thecal and granulosa cells. Our Cochrane review has evaluated the use of metformin for ovulation induction and we concluded that metformin may improve LBR compared with placebo (OR 1.59, 95% CI 1.0–2.51; $I^2 = 0\%$; four studies, 435 women; low-quality evidence) but the evidence was inconclusive when compared with clomiphene. Interestingly, in subgroup analysis, obese patients may fare worse with respect to LBR with metformin compared with clomiphene (OR 0.30, 95% CI 0.17–0.52; two studies, 500 women) [61]. Many women were also found to have gastrointestinal side effects that may limit treatment.

A number of studies have investigated the effects of using insulin sensitizing agents, mainly metformin, on women with PCOS undergoing IVF treatment. A Cochrane review included nine randomized controlled trials, all of which except one used a GnRH agonist protocol [62]. Dose and duration of metformin use was not uniform, ranging from 500 mg twice a day to 850 mg three times a day, for up to 16 weeks prior to hCG trigger. No clear difference in LBR was seen with additional metformin use (OR 1.39, 95% CI 0.81–2.40, five RCTs, 551 women, $I^2 = 52\%$, low-quality evidence); but there was a significant reduction in the incidence of OHSS (OR 0.29, 95% CI 0.18–0.49, eight RCTs, 798 women, moderate-quality evidence) [62]. We performed a large RCT which clearly showed a reduction in the risk of OHSS when metformin was used in the context of a long GnRH agonist protocol [63]. However we did not find any improvement when an antagonist protocol is used [64], yet using metformin resulted in a reduced live birth rate (PLA = 51.6%, MET = 27.6%, 95% CI 0.05–0.40, $P = 0.02$). Although the agonist cycle has now been superseded by the antagonist cycle for women with PCOS, if any women should require an agonist cycle, metformin should be added as an adjunct. For those on an antagonist cycle, metformin confers no advantage and may even be detrimental to the outcome.

Pre-ovulatory trigger

When considering the “pre-ovulatory trigger” the key issue is the protocol used. A significant advantage of the GnRH antagonist protocol is the opportunity to use a GnRH agonist in place of hCG to complete oocyte maturation. The antagonist competitively binds to the GnRH receptor, producing its effect within hours of administration. The agonist can then displace the antagonist from the pituitary receptor, resulting in the release of native luteinizing hormone (LH). Whilst the released LH initiates oocyte nuclear maturation, there is a substantial reduction in surge duration compared with the use of hCG [65]. An adequate amount of LH is required to ensure that optimal luteinization occurs. Early use of the agonist trigger was associated with disappointing pregnancy rates despite a reduction in OHSS. Furthermore, standard luteal phase support is insufficient

to overcome the severe luteal deficiency observed. The pathophysiology of luteal phase insufficiency is secondary to low-level endogenous LH and the shorter half-life of LH. Consequently, the corpus luteum degenerates leaving insufficient progesterone to support early pregnancy development [66]. Modified luteal phase support with either a small dose (1500 IU) of supplementary hCG at the time of oocyte retrieval and/or oestradiol and progesterone overcomes this deficit to a certain extent, leading to similar live birth rates [67]. Use of microdosing of hCG following the GnRH agonist trigger has been suggested as a way to enhance luteal support without increasing the rate of OHSS [67]. Recombinant LH has been tried as an alternative to hCG for final oocyte maturation but without significant benefit [68]. When an agonist trigger is employed, we currently use a combination of a low dose of hCG (1500 units) on the day of oocyte retrieval and luteal support with a combination of progesterone and oestradiol valerate 8 mg daily. Because of potential dysfunction of the HPO axis in PCOS patients, there have been concerns regarding the efficacy of the GnRH agonist trigger in these patients; reassuringly, there is one study that focused on this and refuted this as a problem [69].

Kisspeptins and the connected neuronal network of kisspeptin-neurokinin-B-dynorphin (KNDy) have provided insight into how upstream modulation of the GnRH signal can be harnessed to improve reproductive outcome. Following direct signalling to the GnRH neurone via the kisspeptin receptor, a pulsatile release of GnRH enters the portal circulation. In turn this stimulates pituitary gonadotrophin release, with a preferential secretion of LH and to a lesser extent FSH. By adopting a physiological approach using the hypothalamic endogenous GnRH reserve, a reduction in OHSS may be achieved. Kisspeptin has a half-life of only 28 minutes, in contrast to the extended effect of HCG or even a GnRH trigger. A study to address the optimum dose of Kisspeptin, in a high-risk cohort for OHSS, resulted in high rates of oocyte maturation, high implantation rates, and no cases of clinically significant OHSS [69]. High pregnancy rates were seen throughout the study, with the greatest LBR of 62% following 9.6 nmol/kg kisspeptin-54. Mild OHSS was seen in only four out of the 60 women included.

Ovarian hyperstimulation syndrome

OHSS is the most serious complication of superovulation. Ovarian stimulation leads to increased vascular permeability following release of vasoactive mediators from stimulated ovaries. Vascular endothelial growth factor (VEGF), a potent angiogenic mitogen, is a key mediator of ovarian folliculogenesis and also OHSS. The process of OHSS culminates in fluid shifts from the vascular compartment into the third space, resulting in intravascular dehydration. Symptoms can rapidly progress from mild abdominal distension and nausea to oliguria, ascites, and haematological disturbance. Severe manifestations of OHSS include hepato-renal disturbance, thrombosis, adult respiratory distress syndrome, and even death. The true incidence of OHSS remains unknown, but hospitalization for severe manifestations is low (0.5%–2% of IVF cycles). Clinically significant moderate to severe OHSS affects up to 10% of IVF cycles, whilst milder forms may affect up to a third of cycles [70].

Women with PCOS have an increased incidence of OHSS because of the increased recruitment of gonadotrophin responsive small antral follicles from the primordial pool. This is reflected in the increased levels of anti-Müllerian hormone (AMH) produced

by the many antral follicles of the polycystic ovary. Although the initial response to gonadotrophin stimulation may be slow, once the threshold is reached, the resultant follicular development may be rapid and prolific. An increased expression of VEGF is seen in women with PCOS, and insulin has been shown to augment VEGF secretion [71].

Cabergoline, a dopamine agonist, inhibits phosphorylation of the VEGF receptor, thereby reducing its effects on vascular permeability. Another strategy for reducing OHSS includes starting cabergoline around the time of hCG administration or oocyte collection at a dose of 0.25 mg daily for 10 days [71]. There is no role for laparoscopic ovarian diathermy (LOD, or "drilling") in the reduction of OHSS, as the mechanism of action of LOD, when performed properly, is to sensitize the ovaries to FSH. The only way to reduce ovarian response to FSH after LOD is to destroy the ovary and thereby also destroy valuable oocytes, so this practice should be strongly discouraged.

Attempts have been made to predict those at risk of OHSS, and it seems clear that ultrasound remains the mainstay of both monitoring IVF and predicting the risk of OHSS [72]. If there are more than 19 follicles, we suggest using a GnRH agonist for trigger, and if more than 25 oocytes are collected, or if the patient is symptomatic with fewer oocytes, then elective cryopreservation of embryos is recommended to minimize the risk of OHSS.

In vitro maturation of oocytes for women with PCOS

In vitro maturation (IVM) of oocytes was heralded as a strategy to help eliminate OHSS in women with polycystic ovaries. Oocytes are retrieved from antral follicles in unstimulated or minimally stimulated ovaries. The oocyte then matures *in vitro* in a specially formulated medium for 24–48 hours, before undergoing fertilization with sperm via intracytoplasmic sperm injection. Despite some promising results from early studies, there have not been high-quality trials confirming the viability of this method over standard IVF treatment [73]. Specific clinical and laboratory expertise are required for IVM protocols and so it has not gained widespread popularity. Whilst some groups have reported success [74], a recent large RCT failed to demonstrate any statistically significant differences between the IVM and IVF groups with respect to the occurrence of pregnancy complications, obstetric and perinatal complications, preterm delivery, birthweight, and neonatal complications [75]. In this study of 546 women with polycystic ovaries undergoing IVF, half were randomized to an IVM protocol and the remainder received IVF in an antagonist protocol with a GnRH agonist trigger. Cumulative ongoing pregnancy rates at 12 months after randomization were 44.0% in the IVM group and 62.6% in the IVF group (absolute risk difference –18.7%; 95% CI –27.3%, –10.1%) [75]. OHSS did not occur in the IVM group, versus only two cases in the IVF group. Therefore, whilst an IVM protocol may eliminate OHSS, the rate of ovarian hyperstimulation can be kept to below 0.5% with a carefully conducted antagonist protocol.

Freeze-all embryo strategies

A segmentation approach, with elective freezing of all suitable embryos and embryo transfer in a subsequent frozen embryo replacement cycle, has been advocated as another way to eliminate OHSS [76]. A Cochrane review compared fresh transfer versus a "freeze-all" approach [68] and there was no clear

difference in cumulative LBR (OR 1.09, 95% confidence interval (CI) 0.91–1.31; four trials; 1892 women; $I^2 = 0\%$; moderate-quality evidence). The prevalence of OHSS was lower (but interestingly not eliminated) in the freeze-all group (OR 0.24, 95% CI 0.15–0.38; two trials; 1633 women; $I^2 = 0\%$; low-quality evidence); as was the risk of miscarriage (OR 0.67, 95% CI 0.52–0.86; four trials; 1892 women; $I^2 = 0\%$; low-quality evidence) [76]. This latter point is presumed to be secondary to an improved endometrium without the interference from ovarian hyperstimulation on implantation. Frozen cycles are associated with an increase in pregnancy complications (OR 1.44, 95% CI 1.08–1.92; two trials; 1633 women; low-quality evidence) and there is the inevitable increase in time to pregnancy. Indeed, segmentation and routine use of cryopreserved embryos may increase the incidence of macrosomia, placenta accreta, and pre-eclampsia [76]. A study looking at fresh versus elective frozen cycles in a PCOS population only, confirmed a significant increase in pre-eclampsia with frozen cycles compared with fresh transfer (4.4% vs 1.4%, RR 3.12, 95% CI 1.26–7.73) [77]. An important issue is an apparent increased incidence of stillbirth and neonatal death in the freeze-all group, secondary to prematurity, which was reported in a large RCT of women with polycystic ovaries undergoing IVF [78]. In this study of 1508 women, a frozen-embryo transfer resulted in a higher frequency of live birth than did fresh-embryo transfer (49.3% vs 42.0%), for a rate ratio of 1.17 (95% confidence interval [CI] 1.05–1.31; $P = 0.004$). Women who underwent frozen-embryo transfer also had a lower frequency of pregnancy loss (22.0% vs 32.7%), for a rate ratio of 0.67 (95% CI, 0.54–0.83; $P < 0.001$), and of OHSS (1.3% vs 7.1%), for a rate ratio of 0.19 (95% CI 0.10–0.37; $P < 0.001$), but a higher frequency of preeclampsia (4.4% vs 1.4%). There were also five neonatal deaths in the frozen-embryo group and none in the fresh-embryo group ($P = 0.06$) [78].

These data have led to reserving the use of an elective freeze-all strategy for those who over-respond [79]. More recent large studies have been mixed with respect to outcomes in normal ovulatory, with one indicating that a higher rate of live births can also be achieved [80], whilst another showing no difference [81]. At the time of writing the results of a large UK multicentre RCT (the "E-freeze" study) are awaited [82]. Preliminary data again suggests the main benefit is for those who over respond. Further research is required to qualify the balance between safety, reproductive outcome, and cost, before a segmented-only approach should be adopted for all cycles; nonetheless most prefer this approach for those cycles with a high risk of OHSS.

Summary

In summary, women with PCOS and polycystic ovaries without symptoms require particular care when undergoing IVF in order to minimize the risk of OHSS and optimize outcomes. Low doses of stimulation are required in the context of a GnRH antagonist cycle with the option to freeze-all embryos in those who over respond. Preconception health, in particular attention to nutrition and body weight, is of paramount importance to ensure a healthy outcome for mother, for her pregnancy, and for the future health of the baby.

References

- Stein IF, Leventhal ML. Amenorrhea associated with bilateral polycystic ovaries. Am J Obstet Gynecol. 1935;29(2):181–91. doi: [https://doi.org/10.1016/S0002-9378\(15\)30642-6](https://doi.org/10.1016/S0002-9378(15)30642-6).

2. Balen AH, Morley LC, Misso M, Franks S, Legro RS, Wijeyaratne CN, Stener-Victorin E, Faußer BCJM, Norman RJ, Teede H. The management of anovulatory infertility in women with polycystic ovary syndrome: An analysis of the evidence to support the development of global WHO guidance. *Hum Reprod Update*. 2016;22(6):687–708. doi: <https://doi.org/10.1093/humupd/dmw025>.
3. Faußer B, Tarlatzis B, Chang J, et al. The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod*. 2004;19:41–7.
4. Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L, Piltonen T, Norman RJ. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. *Hum Reprod*. 2018;33(9):1602–18. doi: <https://doi.org/10.1093/humrep/dey256>.
5. Balen AH, Laven JSE, Tan SL, Dewailly D. Ultrasound assessment of the polycystic ovary: International consensus definitions. *Hum Reprod Update*. 2003;9:505–14.
6. Polson DW, Adams J, Wadsworth J, Franks S. Polycystic ovaries—A common finding in normal women. *Lancet*. 1988;1:870–2.
7. Farquhar CM, Birdsall M, Manning P, Mitchell JM, France JT. The prevalence of polycystic ovaries on ultrasound scanning in a population of randomly selected women. *Aust N Z J Obstet Gynaecol*. 1994;34:67–72.
8. Michelmore KF, Balen AH, Dunger DB, Vessey MP. Polycystic ovaries and associated clinical and biochemical features in young women. *Clin Endocrinol (Oxf)*. 1999;51:779–86.
9. Balen AH, Michelmore K. What is polycystic ovary syndrome? Are national views important? *Hum Reprod*. 2002;17:2219–27.
10. Balen AH. The pathogenesis of polycystic ovary syndrome: The enigma unravels. *Lancet*. 1999;354:966–7.
11. Clarke AM, Ledger W, Galletly C, et al. Weight loss results in significant improvement in pregnancy and ovulation rates in anovulatory obese women. *Hum Reprod*. 1995;10:2705–12.
12. Wijeyaratne C, Udayangani D, Balen AH. Ethnic specific PCOS. *Exp Rev Endocrinol Metabol*. 2013;8:71–9.
13. Ünlütürk U, Sezgin E, Yıldız BO. Evolutionary determinants of PCOS: Part 1. *Fertility & Sterility*. 2016;106:33–41.
14. Day F, Karaderi T, Jones MR, et al. Large-scale genome-wide meta-analysis of polycystic ovary syndrome suggests shared genetic architecture for different diagnosis criteria. *PLoS Genet*. 2018 Dec 19;14(12):e1007813. doi: [10.1371/journal.pgen.1007813](https://doi.org/10.1371/journal.pgen.1007813). Erratum in: *PLoS Genet*. 2019 Dec 5;15(12):e1008517.
15. Hiam D, Moreno-Asso A, Teede HJ, Laven JSE, Stepto NK, Moran LJ, Gibson-Helm M. The genetics of polycystic ovary syndrome: An overview of candidate gene systematic reviews and genome-wide association studies. *J Clin Med*. 2019;8(10):1606. doi: [10.3390/jcm8101606](https://doi.org/10.3390/jcm8101606).
16. Cheng C, Zhang H, Zhao Y, Li R, Qiao J. Paternal history of diabetes mellitus and hypertension affects the prevalence and phenotype of PCOS. *J Assist Reprod Genet*. 2015;32(12):1731–9. doi: <https://doi.org/10.1007/s10815-015-0587-y>.
17. Vázquez-Martínez ER, Gómez-Viaiés YI, García-Gómez E, Reyes-Mayoral C, Reyes-Muñoz E, Camacho-Arroyo I, Cerbón M. DNA methylation in the pathogenesis of polycystic ovary syndrome. *Reproduction*. 2019;158(1):R27–R40. doi: <https://doi.org/10.1530/REP-18-0449>.
18. Soubry A, Guo L, Huang Z, Hoyo C, Romanus S, Price T, Murphy SK. Obesity-related DNA methylation at imprinted genes in human sperm: Results from the TIEGER study. *Clinl Epigenetics*. 2016;8(1):51. doi: <https://doi.org/10.1186/s13148-016-0217-2>.
19. Potabattula R, Dittrich M, Schorsch M, Hahn T, Haaf T, El Hajj N. Male obesity effects on sperm and next-generation cord blood DNA methylation. *PLoS ONE*. 2019;14(6):e0218615. doi: <https://doi.org/10.1371/journal.pone.0218615>.
20. Mansour R, El-Faissal Y, Kamel A, Kamal O, Aboulserour G, Aboulghar M, Fahmy I. Increased insulin resistance in men with unexplained infertility. *Reprod Biomed Online*. 2017;35(5):571–5. doi: <https://doi.org/10.1016/j.rbmo.2017.08.020>.
21. Dewailly D, Yding Andersen C, Balen AH, Broekmans FJ, Dilaver N, Griesinger G, Fanchin R, Kelsey T, La Marca A, Lambalk C, Mason H, Nelson S, Visser JA, Wallace HB, Anderson R. The physiology and clinical utility of anti-Müllerian hormone in women. *Hum Reprod Update*. 2014;20:370–85.
22. Risal S, Pei Y, Lu H, et al. Prenatal androgen exposure and transgenerational susceptibility to polycystic ovary syndrome. *Nat Med*. 2019;25(12):1894–904.
23. Padmanabhan V, Veiga-Lopez A. Sheep models of polycystic ovary syndrome phenotype. *Mol Cell Endocrinol*. 2013;373(1-2):8–20. doi: [10.1016/j.mce.2012.10.005](https://doi.org/10.1016/j.mce.2012.10.005).
24. Abbott DH, Nicol LE, Levine JE, Xu N, Goodarzi MO, Dumesic DA. Nonhuman primate models of polycystic ovary syndrome. *Mol Cell Endocrinol*. 2013;373(1-2):21–8. doi: [10.1016/j.mce.2013.01.013](https://doi.org/10.1016/j.mce.2013.01.013).
25. Tata B, Mimouni NEH, Barbotin AL, Malone SA, Loyens A, Pigny P, Dewailly D, Catteau-Jonard S, Sundström-Poromaa I, Piltonen TT, Dal Bello F, Medana C, Prevot V, Clasadonte J, Giacobini P. Elevated prenatal anti-Müllerian hormone reprograms the fetus and induces polycystic ovary syndrome in adulthood. *Nat Med*. 2018;24(6):834–6. doi: [10.1038/s41591-018-0035-5](https://doi.org/10.1038/s41591-018-0035-5).
26. Conti M, Franciosi F. Acquisition of oocyte competence to develop as an embryo: Integrated nuclear and cytoplasmic events. *Hum Reprod Update*. 2018;24(3):245–66. doi: [10.1093/humupd/dmx040](https://doi.org/10.1093/humupd/dmx040).
27. Picton HM, Balen AH. Prenatal androgen exposure and the polycystic ovary syndrome. *Nat Med*. 2019;25:1818–20. doi: <https://doi.org/10.1038/s41591-019-0678-x>
28. Scott R, Zhang M, Seli E. Metabolism of the oocyte and the preimplantation embryo: Implications for assisted reproduction. *Curr Opin Obstet Gynecol*. 2018;30(3):163–70. doi: [10.1097/GCO.0000000000000455](https://doi.org/10.1097/GCO.0000000000000455).
29. Dumesic DA, Padmanabhan V, Abbott DH. Polycystic ovary syndrome and oocyte developmental competence. *Obstet Gynecol Surv*. 2008;63(1):39–48.
30. Weghofer A, Munne S, Chen S, Barad D, Gleicher N. Lack of association between polycystic ovary syndrome and embryonic aneuploidy. *Fertil Steril*. 2007;88(4):900–5.
31. Rice S, Christoforidis N, Gadd C, et al. Impaired insulin-dependent glucose metabolism in granulosalutein cells from anovulatory women with polycystic ovaries. *Hum Reprod*. 2005;20(2):373–81.
32. Skov V, Glintborg D, Knudsen S, et al. Reduced expression of nuclear-encoded genes involved in mitochondrial oxidative metabolism in skeletal muscle of insulin-resistant women with polycystic ovary syndrome. *Diabetes*. 2007;56(9):2349–55.
33. Wood JR, Dumesic DA, Abbott DH, Strauss JF 3rd. Molecular abnormalities in oocytes from women with polycystic ovary syndrome revealed by microarray analysis. *J Clin Endocrinol Metab*. 2007;92(2):705–13.
34. Harris SE, Maruthini D, Tang T, Balen AH, Picton HM. Metabolism and karyotype analysis of oocytes from patients with polycystic ovary syndrome. *Hum Reprod*. 2010;25(9):2305–15.
35. Hickey TE, Legro RS, Norman RJ. Epigenetic modification of the x chromosome influences susceptibility to polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2006;91(7):2789–91.
36. Li Z, Huang H. Epigenetic abnormality: A possible mechanism underlying the fetal origin of polycystic ovary syndrome. *Med Hypotheses*. 2008;70(3):638–42.
37. Boomsma CM, Eijkemans MJC, Hughes EG, Visser GHA, Faußer BCJM, Macklon NS. A meta-analysis of pregnancy outcomes in women with polycystic ovary syndrome. *Hum Reprod Update*. 2006;12(6):673–83.
38. Wax JR. Risks and management of obesity in pregnancy: Current controversies. *Curr Opin Obstet Gynecol*. 2009;21(2):117–23.
39. Palomba S, La Sala GB. Oocyte competence in women with PCOS. *Trends in Endocrinol Metabol*. 2017;28(3):186–98.
40. Qiao J, et al. Extra- and intra-ovarian factors in polycystic ovary syndrome: Impact on oocyte maturation and embryo developmental competence. *Hum Reprod Update*. 2011;17:17–33.

41. Teissier MP, Chable H, Paulhac S, Aubard Y. Comparison of follicle steroidogenesis from normal and polycystic ovaries in women undergoing IVF: Relationship between steroid concentrations, follicle size, oocyte quality and fecundability. *Hum Reprod.* 2000;15(12):2471–7.
42. Westergaard LG, Yding Andersen C, Erb K, et al. Placental protein 14 concentrations in circulation related to hormonal parameters and reproductive outcome in women undergoing IVF/ICSI. *Reprod Biomed Online* 2004;8(1): 91–8.
43. Wood JR, Dumesic DA, Abbott DH, Strauss JF 3rd. Molecular abnormalities in oocytes from women with polycystic ovary syndrome revealed by microarray analysis. *J Clin Endocrinol Metab.* 2007;92(2):705–13.
44. Li Z, Huang H. Epigenetic abnormality: A possible mechanism underlying the fetal origin of polycystic ovary syndrome. *Med Hypotheses.* 2008;70(3):38–42.
45. Anderson R, Groome N, Baird D. Inhibin a and inhibin b in women with polycystic ovarian syndrome during treatment with FSH to induce mono-ovulation. *Clin Endocrinol (Oxf).* 1998;48:577–84.
46. Mason HD, Willis DS, Beard RW, Winston RM, Margara R, Franks S. Estradiol production by granulosa cells of normal and polycystic ovaries (PCO): Relationship to menstrual cycle history and to concentrations of gonadotropins and sex steroids in follicular fluid. *J Clin Endocrinol Metab.* 1994;79:1355.
47. Mills G, Badeghiesh A, Suarthana E, Baghfal H, Dahan MH. Polycystic ovary syndrome as an independent risk factor for gestational diabetes and hypertensive disorders of pregnancy: A population-based study on 9.1 million pregnancies. *Hum Reprod.* 2020;35(7):1666–74.
48. Boomsma CM, Eijkemans MJ, Hughes EG, Visser GH, Fauser BC, Macklon NS. A meta-analysis of pregnancy outcomes in women with polycystic ovary syndrome. *Hum Reprod Update.* 2006;12(6):673–83. doi: [10.1093/humupd/dml036](https://doi.org/10.1093/humupd/dml036).
49. Lim SS, Hutchison SK, Van Ryswyk E, Norman RJ, Teede HJ, Moran LJ. Lifestyle changes in women with polycystic ovary syndrome. *Cochrane Database Syst Rev.* 2019;3:CD007506.
50. Norman RJ, Mol BWJ. Successful weight loss interventions before in vitro fertilization: Fat chance? *Fertil Steril.* 2018;110(4):581–6.
51. Jungheim ES, Lanzendorf SE, Odem RR, Moley KH, Chang AS, Ratts VS. Morbid obesity is associated with lower clinical pregnancy rates after in vitro fertilization in women with polycystic ovary syndrome. *Fertil Steril.* 2009;92(1):256–61.
52. Qiu M, Tao Y, Kuang Y, Wang Y. Effect of body mass index on pregnancy outcomes with the freeze-all strategy in women with polycystic ovarian syndrome. *Fertil Steril.* 2019;112(6):1172–9.
53. Einarsson S, Bergh C, Friberg B, Pinborg A, Klajnbard A, Karlström P-O, Kluge L, Larsson I, Loft A, Mikkelsen-Englund A-L, Stenlöf K, Wistrand A, Thurin-Kjellberg A. Weight reduction intervention for obese infertile women prior to IVF: A randomized controlled trial. *Hum Reprod.* 2017;32(8):1621–30.
54. MBRRAE-UK: Saving lives, improving mothers' care. Available from www.npeu.ox.ac.uk/mbrrace-uk/reports
55. Balen AH, Tan SL, MacDougall J, Jacobs HS. Miscarriage rates following in vitro fertilisation are increased in women with polycystic ovaries and reduced by pituitary desensitisation with buserelin. *Hum Reprod.* 1993;8:959–64.
56. Tarlatzis BC, Koblibianakis EM. GnRH agonists vs antagonists. *Best Pract Res Clin Obstet Gynaecol.* 2007;21(1):57–65.
57. Al-Inany HG, Youssef MA, Ayeleke R, Brown J, Lam W, Broekmans FJ. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev.* 2016;4:CD001750.
58. Farquhar C, Rombauts L, Kremer JA, Lethaby A, Ayeleke RO. Oral Contraceptive pill, progestogen or oestrogen pretreatment for ovarian stimulation protocols for women undergoing assisted reproductive techniques. *Cochrane Database Syst Rev.* 2017 May 25;5(5):CD006109. doi: [10.1002/14651858.CD006109.pub3](https://doi.org/10.1002/14651858.CD006109.pub3).
59. van Wely M, Kwan I, Burt AL, Thomas J, Vail A, Van der Veen F, Al-Inany HG. Recombinant versus urinary gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. *Cochrane Database Syst Rev.* 2011;2:CD005354.
60. Weiss NS, Kostova E, Nahuis M, Mol BWJ, van der Veen F, van Wely M. Gonadotrophins for ovulation induction in women with polycystic ovary syndrome. *Cochrane Database Syst Rev.* 2019;1(1):CD010290.
61. Sharpe A, Morley LC, Tang T, Norman RJ, Balen AH. Metformin for ovulation induction (excluding gonadotrophins) in women with polycystic ovary syndrome. *Cochrane Database Syst Rev.* 2019;12:CD013505.
62. Tso LO, Costello MF, Albuquerque LE, Andriolo RB, Macedo CR. Metformin treatment before and during IVF or ICSI in women with polycystic ovary syndrome. *Cochrane Database Syst Rev.* 2014;11:CD006105.
63. Tang T, Glanville J, Orsi N, Barth JH, Balen AH. The use of metformin for women with PCOS undergoing IVF treatment. *Hum Reprod.* 2006;21(6):1416–25. doi: [10.1093/humrep/del025](https://doi.org/10.1093/humrep/del025).
64. Jacob SL, et al. A short course of metformin does not reduce OHS in a GnRH antagonist cycle for women with PCOS undergoing IVF: A randomised placebo-controlled trial. *Hum Reprod.* 2016;31(12):2756–64.
65. Humaidan P, et al. The luteal phase after GnRH-agonist triggering of ovulation: Present and future perspectives. *Reprod Biomed Online.* 2012;24(2):134–41.
66. Haahr T, Roque M, Esteves SC, Humaidan P. GnRH agonist trigger and LH activity luteal phase support versus hCG trigger and conventional luteal phase support in fresh embryo transfer IVF/ICSI cycles-a systematic PRISMA review and meta-analysis. *Front Endocrinol (Lausanne).* 2017;8:116. doi: [10.3389/fendo.2017.00116](https://doi.org/10.3389/fendo.2017.00116).
67. Andersen CY, Elbaek HO, Alsberg B, Laursen RJ, Povlsen BB, Thomsen L, et al. Daily low-dose hCG stimulation during the luteal phase combined with GnRHa triggered IVF cycles without exogenous progesterone: A proof of concept trial. *Hum Reproduction.* 2015;30(10):2387–95.
68. Youssef MA, Abou-Setta AM, Lam WS. Recombinant versus urinary human chorionic gonadotrophin for final oocyte maturation triggering in IVF and ICSI cycles. *Cochrane Database Syst Rev.* 2016;4(4):CD003719.
69. Abbara A, Jayasena CN, Christopoulos G, et al. Efficacy of kisspeptin-54 to trigger oocyte maturation in women at high risk of ovarian hyperstimulation syndrome (OHSS) during in vitro fertilization (IVF) therapy. *J Clin Endocrinol Metab.* 2015;100(9):3322–31. doi: [10.1210/jc.2015-2332](https://doi.org/10.1210/jc.2015-2332).
70. Mourad S, Brown J, Farquhar C. Interventions for the prevention of OHSS in ART cycles: An overview of Cochrane reviews. *Cochrane Database Syst Rev.* 2017;23(1):CD012103.
71. Peitsidis P, Agrawal R. Role of vascular endothelial growth factor in women with PCO and PCOS: A systematic review. *Reprod Biomed Online.* 2010;20(4):444–52.
72. Kwan I, Bhattacharya S, Woolner A. Monitoring of stimulated cycles in assisted reproduction (IVF and ICSI). *Cochrane Database Syst Rev.* 2021 Apr 12;4(4):CD005289. doi: [10.1002/14651858.CD005289.pub4](https://doi.org/10.1002/14651858.CD005289.pub4).
73. Siristatidis CS, Maheshwari A, Vaidakis D, Bhattacharya S. In vitro maturation in subfertile women with polycystic ovarian syndrome undergoing assisted reproduction. *Cochrane Database Syst Rev.* 2018;(11):CD006606.
74. Mostinckx L, Segers I, Belva F, Buyl R, Santos-Ribeiro S, Blockeel C, Smits J, Anckaert E, Tournaye H, De Vos M. Obstetric and neonatal outcome of ART in patients with polycystic ovary syndrome: IVM of oocytes versus controlled ovarian stimulation. *Hum Reprod.* 2019;34(8):1595–607.
75. Vuong LN, Ho VNA, Ho TM, Dang VQ, Phung TH, Giang NH, Le AH, Pham TD, Wang R, Smits J, Gilchrist RB, Norman RJ, Mol BW. In-vitro maturation of oocytes versus conventional IVF in women

- with infertility and A high antral follicle count: A randomized non-inferiority controlled trial. *Hum Reprod.* 2020;35(11):2537–47. doi: [10.1093/humrep/deaa240](https://doi.org/10.1093/humrep/deaa240).
76. Devroey P, Polyzos NP, Blockeel C. An OHSS-free clinic by segmentation of IVF treatment. *Humin Reprod.* 2011;26(10):2593–7. doi: <https://doi.org/10.1093/humrep/der251>.
77. Wong KM, van Wely M, Mol F, Repping S, Mastenbroek S. Fresh versus frozen embryo transfers in assisted reproduction. *Cochrane Database Syst Rev.* 2017;3(3):CD011184.
78. Chen Z-J, Shi Y, Sun Y, Zhang B, Liang X, Cao Y, Yang J, Liu J, Wei D, Weng N, Tian L, Hao C, Yang D, Zhou F, Shi J, Xu Y, Li J, Yan J, Qin Y, Legro RS. Fresh versus frozen embryos for infertility in the polycystic ovary syndrome. *N E J Med.* 2016;375(6):523–33. doi: <https://doi.org/10.1056/NEJMoa1513873>.
79. Bosdou JK, Venetis CA, Tarlatzis BC, Grimbizis GF, Kolibianakis EM. Higher probability of live-birth in high, but not normal, responders after first frozen-embryo transfer in A freeze-only cycle strategy compared to fresh-embryo transfer: A meta-analysis. *Hum Reprod.* 2019;34(3):491–505. doi: [10.1093/humrep/dey388](https://doi.org/10.1093/humrep/dey388).
80. Wei D, Liu JY, Sun Y, Shi Y, Zhang B, Liu JQ, Tan J, Liang X, Cao Y, Wang Z, Qin Y, Zhao H, Zhou Y, Ren H, Hao G, Ling X, Zhao J, Zhang Y, Qi X, Zhang L, Deng X, Chen X, Zhu Y, Wang X, Tian LF, Lv Q, Ma X, Zhang H, Legro RS, Chen ZJ. Frozen versus fresh single blastocyst transfer in ovulatory women: A multicentre, randomised controlled trial. *Lancet.* 2019 Mar 30;393(10178):1310–18. doi: [10.1016/S0140-6736\(18\)32843-5](https://doi.org/10.1016/S0140-6736(18)32843-5).
81. Stormlund S, Sopa N, Zedeler A, Bogstad J, Prætorius L, Nielsen HS, Kitlinski ML, Skouby SO, Mikkelsen AL, Spangmose AL, Jeppesen JV, Khatibi A, la Cour Freiesleben N, Ziebe S, Polyzos NP, Bergh C, Humaidan P, Andersen AN, Lössl K, Pinborg A. Freeze-all versus fresh blastocyst transfer strategy during in vitro fertilisation in women with regular menstrual cycles: Multicentre randomised controlled trial. *BMJ.* 2020 Aug 5;370:m2519. doi: [10.1136/bmj.m2519](https://doi.org/10.1136/bmj.m2519).
82. Bell JL, Hardy P, Greenland M, Juszczak E, Cole C, Maheshwari A, Bhattacharya S, Linsell L. E-freeze - A randomised controlled trial evaluating the clinical and cost effectiveness of A policy of freezing embryos followed by thawed frozen embryo transfer compared with A policy of fresh embryo transfer, in women undergoing in vitro fertilisation: A statistical analysis plan. *Trials.* 2020 Jun 30;21(1):596. doi: [10.1186/s13063-020-04441-9](https://doi.org/10.1186/s13063-020-04441-9).
83. O'Neill KE, Senapati S, Dokras A. Use of gonadotropin-releasing hormone agonist trigger during in vitro fertilization is associated with similar endocrine profiles and oocyte measures in women with and without polycystic ovary syndrome. *Fertil Steril.* 2015;103(1):264–9.

FERTILITY PRESERVATION STRATEGIES

Claus Yding Andersen and Stine Gry Kristensen

Overview

Fertility preservation focuses on saving gametes in girls and young women who run the risk of losing the entire pool of ovarian follicles, such as by having a cancer or a genetic disease. Future areas include women who wish to delay childbearing [1] or who suffer from endometriosis or have a disposition for premature ovarian insufficiency. Additionally included are hormonal effects that focus on the steroid-producing capacity of follicles and include postponing menopause [2].

For many cancers, the chance of surviving is steadily increasing and is now at around 80%. Many women have started to focus on quality of life aspects after treatment, and the possibility of having their own children is of high priority. Around 2% of women in their reproductive age suffer from invasive cancer and are at risk of ovarian failure after receiving sterilizing chemotherapy and radiotherapy [3]. In contrast to the testis, the ovary is equipped with a fixed number of oocytes without germ stem cells, leaving no possibility for replenishment of the pool of oocytes. Until recently, cryopreserved oocytes or embryos from *in vitro* fertilization (IVF) treatment were considered the only possible options for women to conceive after recovery from a sterilizing cancer treatment. These methods, however, cannot sustain long-term fertility, including support of functioning ovulatory cycles, and are not applicable to prepubertal girls. Further, they require at least two weeks for stimulation, among other factors, which may be incompatible with an urgent cancer treatment. Cryopreservation and transplantation of ovarian tissue overcomes a number of these shortcomings: grafting of cryopreserved ovarian tissue can restore menstrual cyclicity to patients who entered menopause as a consequence of the treatment, and the patient gets the possibility of spontaneous conception [4, 5]. The technique can be performed from one day to another. Moreover, the method is applicable even in prepubertal girls, and ovarian tissue cryopreservation may be performed even in cases where chemotherapy has already been initiated [6], in contrast to IVF treatment.

Fertility preservation in young women and men who have experienced gonadotoxic treatment is now a central topic for professionals and patients [7], and the different strategies in this area will be discussed in this chapter. There is a special focus on cryopreservation of ovarian tissue, as this method is now accepted by the American Society for Reproductive Medicine (ASRM) as a valid method of long-term fertility preservation in girls and women facing gonadotoxic therapy [8], while freezing testicular tissue is still in its infancy, although advances are now being made.

Effects of chemotherapy and radiotherapy on the ovary

Chemotherapy drugs cause a reduction in the number of primordial follicles, diminish ovarian weight, and augment ovarian atrophy. The extent of the damage depends largely on the specific

regimen of chemotherapy used and the age of the patient at treatment [9, 10]. The fact that ovaries of young girls contain a higher number of follicles than ovaries from older women makes them more resistant to chemotherapy and delays the age at which they potentially enter primary ovarian insufficiency (POI) [11].

Alkylating agents such as cyclophosphamide and busulfan are far more gonadotoxic than other chemotherapeutic agents. A study of young cancer patients found that the use of alkylating agents had an odds ratio (OR) of 4.0 for POI, which is significantly higher than when using platinum agents (OR = 1.8), plant alkaloids (OR = 1.2), or antimetabolites (OR <1) [12]. The alkylating agents have been shown to cause extensive loss of primordial follicles in cancer patients [9, 13], and animal studies indicate that this loss is dose-dependent [14]. The alkylating agent cyclophosphamide is a cell cycle-non-specific drug, and as such it is more cytotoxic to the ovaries than cell cycle-specific drugs, as it may harm both resting and dividing cells. Studies in mice have shown massive apoptosis observed in the granulosa cells of growing, though not resting, follicles of cyclophosphamide-treated mice [10]. However, interestingly, the same study demonstrated both a decrease in primordial follicles and an increase in early growing follicles, which suggest that cyclophosphamide actually activated the growth of the resting primordial follicle pool in mice, resulting in loss of the ovarian reserve [10].

Radiotherapy interrupts the normal cellular proliferation cycle and causes extensive cell damage. However, despite postnatal oocytes being mitotically inactive, they are still highly susceptible to the damage caused by radiotherapy. The prepubertal ovary is less vulnerable than in later reproductive life simply because of higher numbers of oocytes, but the risk of POI after abdominal radiotherapy is still considerable [15]. It has been estimated that a total radiation exposure of 20 Gy fractionated over six weeks in younger women and children produces sterility with 95% confidence [16]. Finally, the high-dose chemotherapies and radiotherapies used prior to bone marrow transplantation (BMT) leave the vast majority of patients without ovarian function and fertility [12].

Candidates for fertility-preserving methods

Estimating the risk of POI for any given patient is difficult and dependent on a number of factors, including age, disease, stage of disease, and the fact that the planned chemotherapy treatment may change in relation to the specific response obtained as a result of the treatment given [17, 18]. To help physicians evaluate each patient, selection criteria such as the Edinburgh criteria can be used for guidance [19]. In the Danish fertility preservation program, the criteria are:

- A more than 50% risk of post-treatment infertility
- An estimated greater than 50% chance of surviving five years after diagnosis
- An upper age limit of around 35 years, depending on the relative number of antral follicles and the concentration of AMH

Collectively, selection criteria should merely be used as guidance and not exact rules, as each woman should have an individual assessment of her ovarian reserve as well as her risk of POI. Risk is a relative term and some may find that for instance a risk of POI on 20% is low and therefore decline the procedure, whereas others may find the procedure worthwhile given the cost and the effort they need to provide.

Risk assessment

Most antineoplastic treatments in childhood are not hazardous to the immediate ovarian function, although they may reduce the future ovarian function and fertility potential. However, some treatments and cancer diagnoses are associated with a high risk of POI, and in these cases, fertility-preserving methods should be discussed with and offered to the woman or, in case of a young girl, together with her parents [20]. The different fertility-preserving techniques' pros and cons and their relevance to girls and young women according to the planned treatment are presented in Table 65.1. Patients with an almost 100% risk of POI are those for whom BMT is planned and those receiving abdominal radiation. In cases where high-dose chemotherapy is planned, the indication for fertility preservation should be evaluated individually in relation to the planned dose and type of drug used. In patients who have already received a relatively mild chemotherapy because of a malignancy and who later experience a relapse, cryopreservation of ovarian tissue may be considered before initiating a new round of chemotherapy, which usually includes more aggressive and gonadotoxic treatment regimens [20]. For a more detailed description of the risk of treatment-related infertility with the main specific anticancer therapies, see Lambertini et al. [18].

Fertility preservation was initially indicated only for cancer patients receiving sterilizing chemotherapy; however, today indications cover patients receiving gonadotoxic chemotherapy for other systematic illnesses, such as autoimmune diseases, in some patients undergoing oophorectomy for benign ovarian conditions, and in patients with genetic diseases that causes follicular depletion in the early years, such as Turner syndrome and galactosemia.

Current options for the preservation of female fertility

When a patient faces a substantial risk of POI, the different methods for fertility preservation, their advantages and disadvantages, their efficiency, and the possible experimental nature of the treatment need to be taken into consideration (Figure 65.1).

Hormonal suppression

It has been suggested that co-treatment with gonadotropin-releasing hormone (GnRH) analogues should protect the ovaries from the harmful effects of chemotherapy [21, 22]. Currently, there is no solid evidence to show a beneficial effect of GnRH analogues [23, 24].

Cryopreservation of mature oocytes or embryos

Methods for cryopreservation of mature oocytes and embryos derived from couples undergoing IVF treatment are now standard and represent an effective method for preserving female fertility. In the infertile population, pregnancy rates between fresh and frozen-thawed oocytes or embryos are now almost the same. Among women with cancer, one retrospective study reported a live birth rate of 44.4% [25].

The primary drawbacks to IVF include the time required, cost, and risk of ovarian hyperstimulation syndrome. Moreover, patients should be aware that around 20 vitrified oocytes are required to achieve a live birth, as the live birth rate per vitrified oocyte (oocyte donation) is 5.7% in the most experienced teams in the world [26]. Ovarian stimulation can now be performed with a random start anywhere in the menstrual cycle, but still the IVF procedure will cause a delay in initiation of treatment of two to four weeks [27, 28].

Cryopreservation of ovarian tissue

The success of ovarian cryopreservation is based on the high cryopreservation tolerance of small (resting) primordial follicles in contrast to the vulnerable, larger, growing follicles. The vast majority of primordial follicles are located in the outermost

TABLE 65.1 Fertility Preserving Measures Applicable to Female Patients with a Malignant Diagnosis—Pros and Cons

Method	Planned Treatment	Age Group	Mode of Obtaining	Advantages	Disadvantages
Oophorectomy	Abdominal radiation	P– girls P+ girls Adult women	Spontaneous or IVF	Standard procedure	Scatter radiation
Cryopreservation of oocytes and embryos	BMT, abdominal radiation, and high-dose AA	(P+ girls?) Adult women	Fertilization of oocytes and/or embryo transfer	Established technique	May incur delay Requires sperm Fixed fertility potential Not appropriate for P– girls
Cryopreservation of ovarian tissue	BMT, abdominal radiation, and high-dose AA	P– girls P+ girls Adult women	Spontaneous or IVF after transplantation of frozen-thawed tissue	Minimal delay Restores ovarian function → spontaneous and repeated conception No lower age limit	Requires surgery Risk of malignant cell contamination Efficacy unknown

Source: Table modified from Schmidt KT et al. *BJOG* 2010;117:163–74.

Abbreviations: AA, alkylating agents; BMT, bone marrow transplantation; IVF, *in vitro* fertilization; P–, prepubertal; P+, post-pubertal.

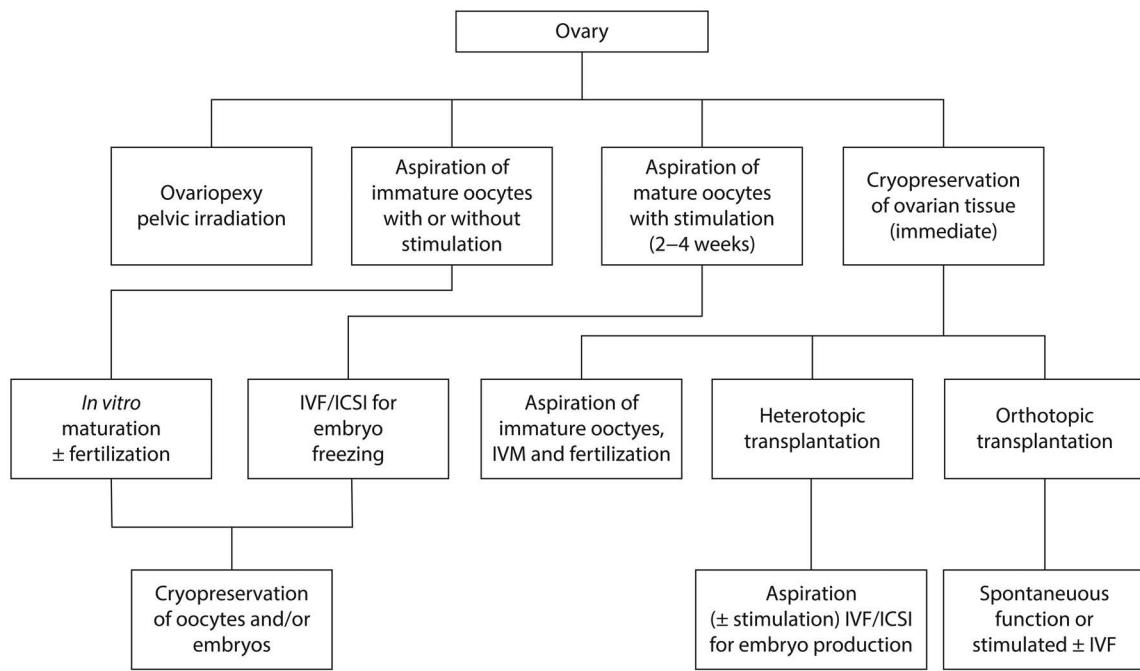


FIGURE 65.1 Options for fertility preservation in women. Abbreviations: IVM, *in vitro* maturation; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

1 or 2 mm of the ovarian cortex, which is relatively easy to isolate from the rest of the ovarian tissue. When the ovarian cortex has been frozen, it can be stored for years in liquid nitrogen, allowing time for the patient to recover. After the patient is cured, some of the cryopreserved tissue can be re-transplanted to those who entered menopause, and the ovarian grafts are able to re-establish a cyclic endocrine hormone milieu, including appropriate conditions for conception, gestation, and parturition, possibly via IVF–embryo transfer [29–31].

In the future, cryopreservation of ovarian tissue might be associated with the aspiration of small antral follicles followed by *in vitro* maturation (IVM), which could potentially offer the patient an additional chance of becoming pregnant [26, 32]. Immature oocytes can be collected from antral follicles in the ovarian tissue or found in the dissection medium at the time of the cryopreservation procedure, matured *in vitro*, and then cryopreserved [33, 34]. The first live birth resulting from a cryopreserved embryo obtained from *in vitro*-matured oocytes collected after oophorectomy was recently reported [35], as was the second clinical pregnancy [36].

Technical aspects of ovarian tissue cryopreservation

The Danish Fertility Preservation Program was initiated in 1999. Since then, more than 1400 patients have had ovarian tissue cryopreserved for clinical purposes, and, currently, 16–20 ovaries per year per million inhabitants are frozen. Since 2003, 139 autotransplantations have been performed in 107 women, resulting in more than 40 clinical pregnancies, of which more than 30 resulted in the birth of healthy babies [37–39], two legal abortions [40], and two second-trimester miscarriages. Thus, the Danish protocol for ovarian tissue cryopreservation has proven quite

robust, and is now an integrated and a well-established method of fertility preservation in the Danish healthcare system.

The Danish protocol

In almost all Danish patients, an entire ovary is removed surgically via laparoscopy (Figure 65.2a). The advantages of removing the whole ovary are the minimized risk of postoperative complications and the possibility of prolonging the window of possible fertility by repeated transplantations. Women with a single ovary may have slightly elevated serum follicle-stimulating hormone (FSH) concentrations [41], but appear to maintain a normal fertility potential either through natural conception or via IVF [42]. However, the decision to cryopreserve one whole ovary in contrast to parts of it remains a matter of debate [43].

The ovary is transported to the cryopreservation facility and, at a sterile bench, the ovary is placed in a 10-cm Petri dish containing 20 mL isotonic saline solution, and the cortex is manually isolated using hooked forceps and scalpels (Figure 65.3). When all medullary tissue is removed and the cortex has been trimmed to a thickness of 1–2 mm (Figure 65.2b), it is cut into 5 × 5 × 1-mm pieces (Figure 65.2c). During the trimming procedure, the tissue is rinsed several times in an isotonic saline solution. The pieces are transferred to a 50-mL plastic tube containing 30 mL freezing solution (0.1 mol/L sucrose, 1.5 mol/L ethylene glycol, and 10 mg/mL human serum albumin [HSA] in phosphate-buffered saline [PBS]), and equilibrated for approximately 25 minutes at 1–2°C on a tilting table. The fragments of cortex are transferred individually to 1.8-mL cryovials (Nunc A/S, Roskilde, Denmark) using sterile forceps, each containing 1 mL fresh freezing solution, and these are cryopreserved using a programmable freezer (Planer 360-1.7, Planer Ltd, Middlesex, UK) (Figure 65.2d). The following program is used: starting temperature 1°C, then –2°C/minute to –9°C, five minutes of soaking, followed by manual seeding for ice crystal induction, –0.3°C/minute to –40°C,



FIGURE 65.2 Cryopreservation of human ovarian tissue for fertility preservation. (a) One ovary or part of an ovary is surgically removed. (b) The medulla is removed and the cortex is trimmed to a thickness of 1–2 mm. (c) The cortex is then cut into pieces of $5 \times 5 \times 1$ mm. The pieces of cortex equilibrate in a cold freezing solution for 25 minutes on ice. (d) The cortex pieces are transferred to individual cryotubes, manually seeded, and slow frozen in liquid nitrogen with a programmable Planer Freezer.

–10°C/minute to –140°C, and then directly into liquid nitrogen. From the moment the tissue enters the freezing solution and until initiation of the cryo-program, exactly 30 minutes elapses and the temperature is constantly kept at around 1–2°C. Following freezing, the tubes are sealed in a second plastic holster (double

sealing) (Figure 65.4a), and half of the tissue is long-term stored in each of two separate nitrogen tanks (Figure 65.4b). During the procurement of the cortical tissue, a small piece of cortex is taken for histology and used to estimate the follicular density.

Quality control by xenotransplantation of human ovarian tissue

To qualitatively assess follicle survival following freezing, frozen-thawed ovarian cortical biopsies from 42 women were transplanted under the skin of oophorectomized immunodeficient mice a total of 49 times in our program [30]. From these women, 36 had a malignant diagnosis prior to cryopreservation: breast cancer ($n = 9$), Hodgkin's and non-Hodgkin's lymphoma ($n = 9$), leukaemia (acute lymphoblastic, chronic myeloid, and acute myeloid; $n = 7$), sarcoma ($n = 5$), and miscellaneous ($n = 6$). The mice were killed after four weeks (Figure 65.5a). Histological evaluation showed healthy primordial follicles in all of the cortical biopsies and confirmed that follicular viability was maintained after thawing (Figure 65.5b). Transplantation of frozen-thawed ovarian tissue to immunodeficient mice is still considered to be the best way of evaluating the survival of follicles.

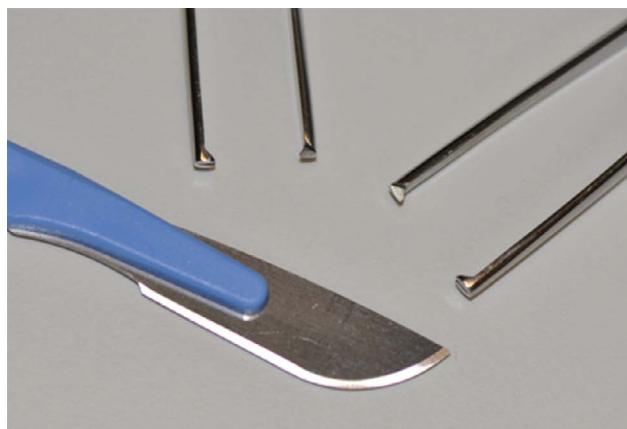


FIGURE 65.3 Instruments used for preparation of the ovarian cortex. Hooked forceps ensure a firm hold on the ovarian tissue during dissection, and a scalpel with a long cutting edge enables a smooth trimming of the cortex.

Slow freezing versus vitrification

The most widely used protocol for ovarian cryopreservation is the slow-freezing technique [44–46], and up until now, all children born (but a few) have resulted from slow-frozen cortical tissue [39, 47, 48]. Two techniques are currently being tested as alternatives

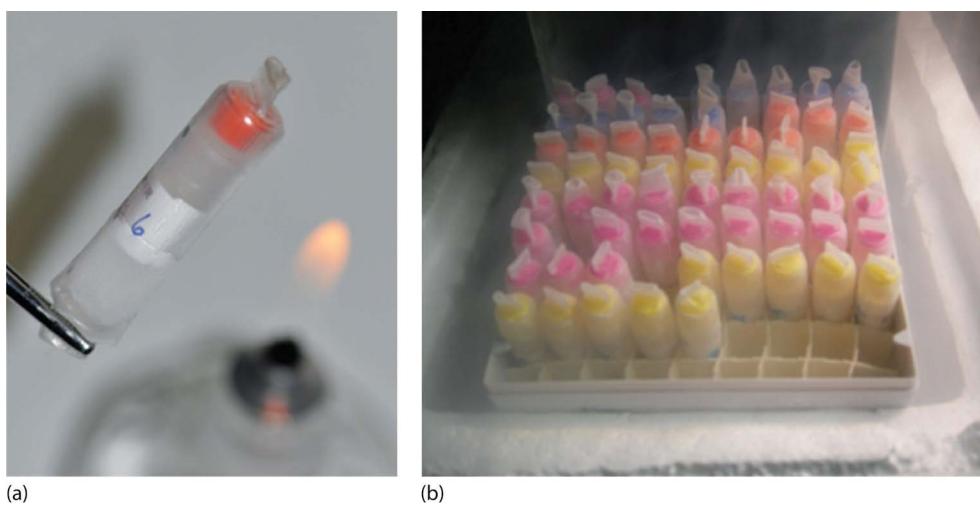


FIGURE 65.4 Dobbelt sealing and storage of cryo-tubes. (a) Once frozen, the cryo-tubes containing the ovarian tissue are double sealed in CryoFlex, (b) before long-term storage. Notice the different colour codes for each patient.

to the slow-freezing method. The first is vitrification, in which the tissue is exposed to high concentrations of cryo-protectants for a short time and immediately plunged in liquid nitrogen similar to the now widely used method for storing oocytes and embryos. Compared with slow freezing, vitrification has been found by some researchers to be associated with improved maintenance of ovarian follicular and stromal structures, as well as increased follicle survival rates [49–51]. However, others find superior results using slow freezing [52, 53], and it is currently questionable whether vitrification offers any significant clinical benefit. The second alternative is whole-ovary freezing, in which the cryoprotectants are introduced through the vascular pedicles *in vitro* followed by cryopreservation [54], and this approach may avoid

the ischemia-induced follicle loss that occurs in connection with transplantation because anastomosis of ovarian vessels ensures a rapid blood supply [55]. However, currently, the ovarian vessels seem to become damaged during the freezing process and this method is not currently used clinically.

Transportation of ovarian tissue prior to cryopreservation

Ovarian tissues remain viable after transportation for up to five hours on ice prior to freezing [30, 56]. This allows hospitals without cryopreservation expertise to treat women locally for the cancer disease and just send the ovarian tissue to the centre that performs cryopreservation. This facilitates quality control,

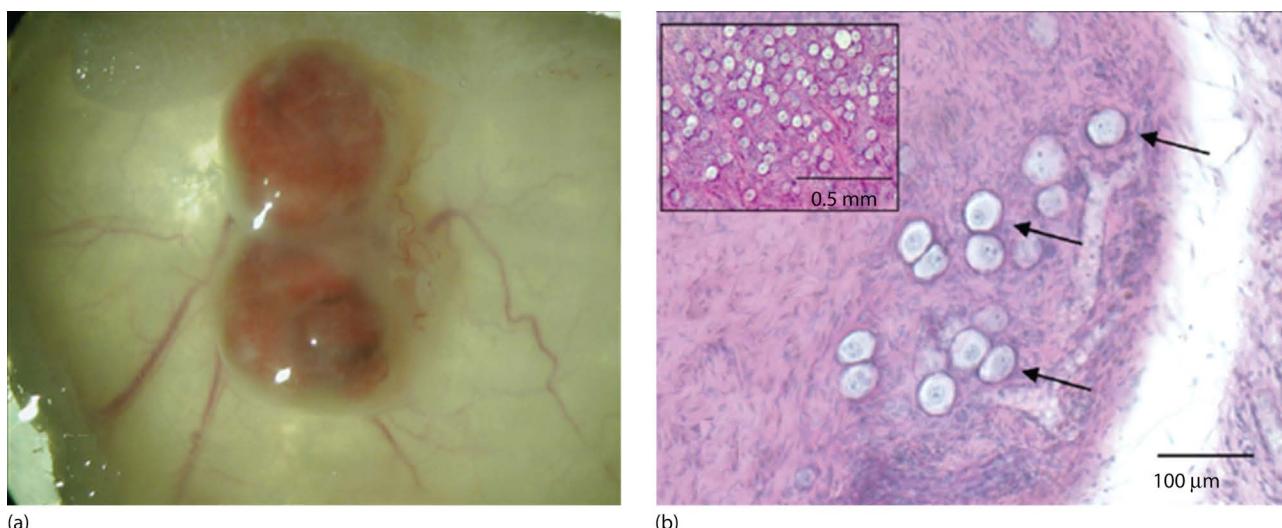


FIGURE 65.5 (a) Two pieces of frozen-thawed human ovarian cortex (5×5 mm) transplanted under the skin of an oophorectomized mouse. After two weeks, macroscopically visible revascularization was established. (b) Human ovarian cortex kept on ice for 20 hours prior to freezing. Thawed tissue was transplanted under the skin of an oophorectomized mouse for four weeks. Histology showed healthy primordial follicles (arrows) surrounded by small blood vessels. Insert shows histology of the fresh sample. (From Rosendahl M et al. *Reprod Biomed Online* 2011;22:162–71.)

proper equipment, and personnel to fulfil clinical, legal, and scientific standards required for properly conducting the procedure. The feasibility of centralized cryo-banking has been proven by the Danish experience of transporting ovarian tissue prior to freezing, and these principles have now been introduced in Germany (where overnight transport is performed) and many other countries [5]. We have demonstrated good follicle survival after freezing and transplantation of human ovarian cortex to ovariectomized immunodeficient mice for a period of four weeks following a transport period of 20 hours on ice prior to cryopreservation (Figure 65.5b) [30], and recent German results show that overnight transportation of tissue before freezing results in live births [57].

Autotransplantation of cryopreserved ovarian tissue

Although ovarian tissue from thousands of girls and women has been cryopreserved, globally, results from transplantation are accumulating at a slow pace. Usually, the patient needs at least two years for cure before receiving transplantation. Furthermore, fortunately, merely half of the women who had one ovary cryopreserved actually entered menopause immediately or shortly after termination of treatment [58]. The number of re-implantations performed worldwide is not known; however, it is estimated to have approached around 1000 by mid-2022.

Thawing of cryopreserved ovarian tissue

Thawing consists of a three-step procedure, each lasting 10 minutes (Figure 65.6). The vials containing the frozen tissue are placed in a 37°C water bath. Immediately after the solution liquifies, the cortical tissue is removed and placed in the first thawing medium (0.75 mol/L ethylene glycol, 0.25 mol/L sucrose, and 10 mg/mL HSA in PBS) and then with sterile forceps moved to the second medium (0.25 mol/L sucrose and 10 mg/mL HSA in PBS) on a tilting table at room temperature. For the last 10 minutes of thawing, the tissue is transferred to PBS with 10 mg/mL HSA in

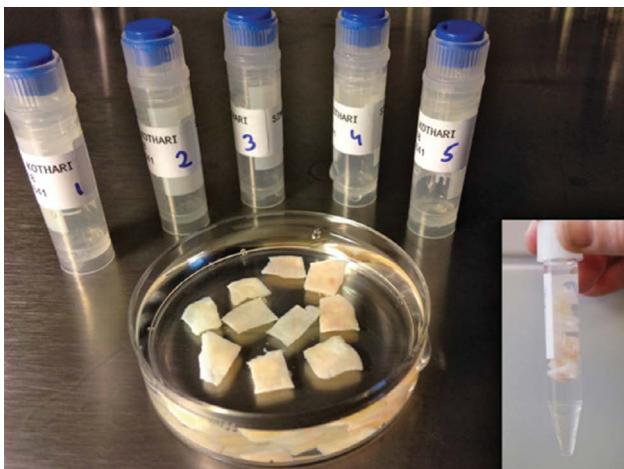


FIGURE 65.6 Three-step thawing procedure. Pieces of cortex are transferred to thawing solutions with decreasing concentrations of cryo-protectant. The inset shows the final step of thawing, and the tissue is subsequently brought to the operating theatre for transplantation.

a 10-mL plastic tube (Figure 65.6, insert), which is brought to the operating theatre for immediate re-implantation. The period of time between transplantation and revascularization of the tissue appears to be critical to follicle survival, since 60%–70% of follicles have been found to be lost in connection with transplants in sheep [59], whereas only a small fraction is lost due to the actual cryopreservation procedure.

Orthotopic and heterotopic transplantation

In most Danish patients, transplantation has been performed as a combined laparoscopy/mini-laparotomy to subcortical pockets of the remaining menopausal ovary [60]. Under general anaesthesia, a 50-mm surgical incision to the lower abdomen is performed, and the remaining ovary is mobilized laparoscopically and made available on the surface. Longitudinal incisions in the ovarian cortex are made, thus creating small pockets just below the cortex on each side of the ovary (Figures 65.7a and 65.7b). The fragments are aligned next to one another in the pockets with the cortex side outward [60]. Normally, 6–10 pieces of cortex can be positioned onto the remaining ovary depending on the size of the ovary. Whenever possible, the tissue is transplanted under the cortex of the remaining ovary left *in situ* (orthotopic transplantation; Figures 65.7a and 65.7b); however, in some cases in which the remaining ovary has been removed or the ovarian volume is significantly reduced, it is necessary to transplant the tissue to peritoneal pockets on the anterior abdominal wall or to the lateral pelvic wall (peritoneal orthotopic or heterotopic transplantation; Figures 65.7c and 65.7d) [61, 62].

Restoration of ovarian activity and outcomes

Ovarian activity is normally restored within 3.5–6.5 months post-grafting, which concurs with the period of follicle growth from the early developmental stages to the antral stage [30, 31, 63]. FSH concentrations measured after the first transplants in 12 Danish patients show that FSH remains high for a short period after transplantation until follicular growth reaches a stage in which oestradiol and inhibin B are secreted, and then starts to decline towards premenopausal concentrations (Figure 65.8).

In 2013, three different European centres (from Belgium, Denmark, and Spain) collected and evaluated the results of 60 orthotopic re-implantation cases [31]. Fifty-one of the 60 patients were followed up over six months later. The study demonstrated that 93% of the women showed restoration of ovarian activity. Eleven of these 51 patients became pregnant and six had already given birth to 12 healthy children at the time of the follow-up. In addition, more than 50% of the women who became pregnant were able to conceive naturally, which favours orthotopic transplantation. Moreover, the age of patients at the time of cryopreservation has previously been reported to be a predictive factor for pregnancy [64], and the majority of pregnant women were actually under the age of 30 years.

Furthermore, it has now been shown that immature ovarian tissue frozen before puberty, after transplantation responds to endogenous gonadotropin stimulation in a way similar to ovarian tissue harvested from adult women with a functioning ovary at the time of freezing [65]. In addition, some of these women have now conceived, which resulted in healthy babies [65].

In addition, two groups have been able to induce puberty by re-implanting frozen-thawed prepubertal ovarian tissue in two

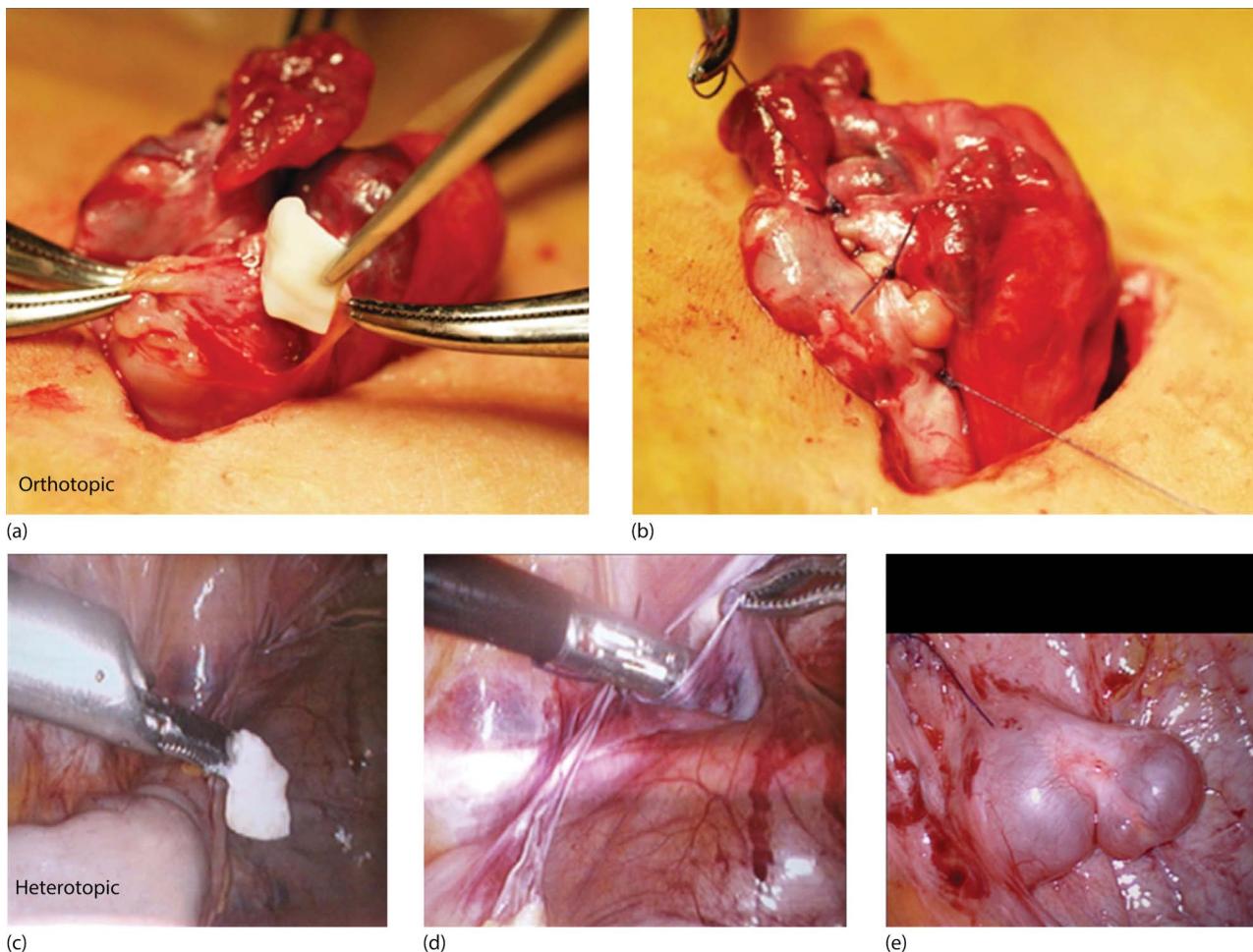


FIGURE 65.7 Transplantation of cryopreserved ovarian tissue. (a and b) Orthotopic transplantation: pieces of thawed ovarian cortex being transplanted in a subcortical pocket in the *in situ* ovary. (c and d) Heterotopic transplantation: pieces of thawed ovarian cortex being transplanted in a subperitoneal pocket corresponding to the pelvic wall. (e) Two human antral follicles at a heterotopic graft site several years after transplantation of thawed ovarian tissue.

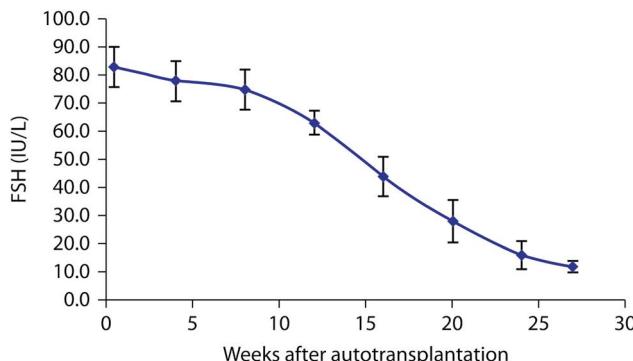


FIGURE 65.8 Restoration of ovarian function serum levels of FSH (IU/L) in 12 Danish patients after autotransplantation of frozen-thawed ovarian tissue (mean \pm standard error of the mean). Abbreviation: FSH, follicle-stimulating hormone.

young girls [66, 67], which demonstrates the wide range of possibilities this method offers.

Pregnancy and live birth rate

Many cases of re-implantation of cryopreserved ovarian tissue have been performed throughout the world and, to date, almost 200 live births have been reported in peer-reviewed journals and abstracts of congresses [39, 68–70]. The worldwide expansion of this technique has now resulted in an endorsement from ASRM and its application in routine clinical practice albeit in specialized centres. In the series of 60 reported live births, the number of re-implantations performed in each centre is unknown, as well as most of the intrinsic ovarian activity before grafting, which makes it impossible to estimate a pregnancy rate [68, 71]. Recently, in 2021, results from five centres were collected to evaluate a series of 285 transplantations [72]. In this large series, the proportion of women who conceived was 26% ($n = 75$). Because the number

of transplantations (the denominator) is known, this information is highly relevant and based on evidence. Two women delivered three babies each, proving the efficacy of the technique, as well as the possibility of conceiving naturally several times after only one procedure [31, 71].

In connection with IVF, it should be pointed out that in series published by Andersen et al. [29], Dolmans et al. [73], and Meirow et al. [74], empty follicle rates as high as 29%–35% were observed during the IVF procedure, and the percentage of immature or degenerated oocytes is much higher (37%) in patients with frozen-thawed transplanted tissue than in the general population undergoing intracytoplasmic sperm injection (ICSI) [73]. In the recent study from Dolmans and co-workers [72], it was also demonstrated that IVF in this group of patients is difficult and all should be considered as poor ovarian response patients. In a study by Greve and co-workers, 12 women who had thawed ovarian tissue transplanted received assisted reproductive techniques, and the outcome per cycle was a pregnancy rate of 6.9% and a live birth rate of 3.8% [75]. It was suggested that the poor outcome reflected reduced follicular selection, rather than aged or damaged oocytes.

Longevity of the grafts

In the Danish cohort, the longevity of the transplants was found to be between nine months and nine years and still functioning [38, 75, 76]. Figure 65.7e shows two human antral follicles at a heterotopic graft site several years after transplantation of ovarian tissue, demonstrating successful grafting and integration of the tissue. Other studies have shown that the transplanted grafts have a mean longevity of approximately four to five years when the follicular density was well preserved [26]. Given that the longevity of the tissue is good and that, in many cases, the women have enough tissue stored for two to three transplantations, the cryopreserved tissue could be enough to restore endocrine function until the natural age of menopause.

Evaluation of ovarian tissue for residual disease

There may be a risk of reintroducing the original cancer in connection with transplantation of ovarian tissue that is removed before the patients receive chemotherapy. However, ovarian tissue cryopreservation is usually only offered to patients with a high chance of long-term survival, and these patients will typically have low-stage and limited disease, with a minimal risk of dissemination and ovarian involvement [26, 30]. Nevertheless, one exception is those patients who have haematological malignancies, where ovarian involvement cannot be excluded.

To minimize risk of grafting ovarian tissue to cancer survivors, a variety of different techniques may be used either separately or in combination [77]: (i) the surgeon performing excision of the tissue should observe for possible gross pathology near and on the ovaries; (ii) before re-implantation, a piece of the frozen-thawed tissue can be evaluated by histology and immunohistochemistry using the markers that characterized the original tumour; (iii) evaluation by reverse transcription and quantitative polymerase chain reaction can be carried out for specific cancer markers; and (iv) immunodeficient mice can be transplanted with pieces of ovarian tissue and kept for four to six months to detect whether the original human cancer develops. If the mice do not develop cancer, it cannot be excluded that the tissue contains tumour cells, as some human cancers do

not grow well in mice. However, even though one ovarian piece has been evaluated to be risk free, it is impossible to completely conclude that other pieces from the same patient might not be harbouring malignant cells and thereby lead to a relapse of the oncological disease.

Risk of malignant cell contamination

Numerous studies have been conducted on the risk of cell contamination by re-implanting cryopreserved ovarian tissue. In 2013, three independent groups carried out systematic reviews of the literature. All three reviews concluded that the highest risk of reintroducing cancer cells via transplantation of cryopreserved ovarian tissue was for leukemic patients [78–80]. Additionally, some of the studies estimated that there was a moderate risk of transplantation for any of the gastrointestinal cancers [78, 80]. The most reassuring data were found in relation to transplantation of patients surviving lymphoma. All three studies concluded that there was a low risk of metastasis in Hodgkin's lymphoma [78–80]. However, reassuring, a review of the literature identified a total of 264 transplantations performed to women with a former malignant diagnosis in whom relapse due to the ovarian tissue did not happen [69], results that have been confirmed in 2021 [72].

Further, in relation to patients with leukaemia, two *in vivo* studies from Denmark and Belgium have found that immunodeficient mice with xenotransplanted tissue from patients in complete remission at the time of tissue collection did not develop leukaemia. These studies concluded that collection of ovarian tissue from patients with leukaemia should be done when they are in complete remission [81, 82]. Even though these results are encouraging for continued storage of tissue from leukaemic patients, there is no established method to regain fertility for patients who suffered from leukaemia, but in the future, isolated follicle transplantation or IVM [83, 84] may become possible.

Most importantly, no relapses have been reported at any graft sites. However, one case report documented a relapse concerning the recurrence of a granulosa cell tumour, but no evidence of tumour was found at the graft site [85]. This could suggest that ovarian diseases may require stricter precautions.

Preservation of male fertility

Subfertility affects approximately 15% of all couples, and a severe male factor is identified in 17% of couples who are affected by subfertility. While the aetiology of a severe male factor infertility remains largely unknown, prior gonadotoxic treatment and genomic aberrations have been associated with this type of subfertility [86].

Effects of chemotherapy and radiotherapy on the testis

The testis has been shown to be highly susceptible to the toxic effects of irradiation and chemotherapy at all stages of life [87]. The impact of combination chemotherapy on the spermatogenic epithelium is dependent on the type and dosage of the drugs used [87–90]. The threshold dose of cyclophosphamide in relation to infertility has been estimated to be between 7.5 and 9 g/m² [91, 92], and in post-pubertal boys to be 10 g/m² [93]. In the prepubertal testis, germline stem cells are acutely and dose-dependently depleted following radiation exposure [94, 95]. Doses of more than 6 Gy are able to deplete the spermatogonial stem cell (SSC) pool and lead

to permanent infertility [96, 97]. Recovery of spermatogenesis can occur from the remaining stem cells, and relies on the type, dose, and fractionation of cytotoxic drugs and irradiation [98].

Current options for the preservation of male fertility

Recently, comprehensive reviews on male fertility preservation have been published [99]. Cryopreservation of ejaculated sperm is the routinely used tool for fertility preservation in adult male patients [100]. Success rates in achieving a pregnancy using cryopreserved sperm have greatly improved by ICSI [101]. All pubertal boys with testis volumes above 10–12 mL are encouraged to donate a semen sample prior to cancer therapy [100, 101]. Alternatively, electroejaculation, penile vibratory stimulation, search for spermatozoa in urine sample, or testicular sperm extraction from a biopsy can be used as sources for retrieving spermatozoa for boys who are unable to ejaculate [102]. Since prepubertal boys cannot benefit from sperm banking, and cryopreserved samples are finite resources that do not offer the possibility of restoring natural fertility, a potential alternative strategy for preserving their fertility is cryopreservation of testicular tissue and SSCs prior to cancer treatment [103]. This application involves enzymatic isolation of SSCs from the frozen-thawed testicular biopsy, *in vitro* propagation, and transplantation of SSCs into the seminiferous tubules via the efferent duct or rete testis [104, 105]. Upon SSC transplantation, SSCs migrate to the basement membrane of the seminiferous tubules, colonize the epithelium, and undergo self-renewal and differentiation so that permanent spermatogenesis is established, which should allow natural conception without further fertility treatment.

Several protocols have already been developed for the cryopreservation of cell suspensions and testicular fragments from adult and cryptorchid testes using propanediol, glycerol, ethylene glycol, or dimethyl sulfoxide [94, 106–108].

Fertility restoration by testicular grafting and transplantation of SSCs

Prepubertal testicular tissue from different species (mice, hamsters, and monkeys) survives freezing surprisingly well and, after xenografting, is able to support sperm production, which can be retrieved from the tissue for assisted reproductive technique procedures [109]. However, no report of successful testicular autografting in men has been published yet.

SSC injection is considered the most promising tool for fertility restoration in prepubertal cancer patients. In mice, germ cell transplantation was successfully performed for the first time in 1994, when microinjection of spermatogonia into the seminiferous tubules prompted germ cell development up to complete spermatogenesis [110]. Due to differences in anatomy and consistency and the larger testis size, injection of SSCs via the rete testis has proved to be a better treatment site for species such as cattle, primates, and humans [111]. In the context of human fertility restoration, adult and prepubertal human SSCs have been successfully grown *in vitro* without losing their stem cell capacity or ability to colonize the seminiferous tubules upon xenotransplantation [112, 113].

Recently, rhesus monkey SSCs have been injected under slow constant pressure into the rete testis under ultrasound guidance, and sperm cells that were able to fertilize oocytes by ICSI were found in the ejaculate of recipients [114]. This study in non-human primates is of course an important milestone towards using SSCs to restore human fertility; however, it remains vitally important to prove that the epigenetic programming and stability of SSCs

are not compromised following cryopreservation, culture, and transplantation in humans [115].

Conclusions

Both established and experimental therapies can now be used to allow young women and men to overcome the infertility that may result from gonadotoxic treatment. Ovarian tissue cryopreservation is becoming a well-established technique for fertility preservation worldwide, and more than 200 healthy children have been born so far using this approach. The number of transplantsations is increasing as women survive their illnesses and return to get their fertility restored. The longevity of the grafts is surprisingly long in some cases, lasting up to nine years and still functioning, thereby showing the strength of this technique. In men, cryopreservation of sperm is the gold standard procedure for preserving fertility, and young boys and men can have their testicular tissue cryopreserved with good results, but strategies for transplantation still need to be established in order to restore fertility.

Most importantly, if there is a risk of gonadal damage and fertility loss, patients should be referred to the infertility specialist by haematologists and oncologists before gonadotoxic treatment is initiated in order to receive the proper counselling on the available fertility preservation strategies.

Future aspects

For the future, new strategies should be optimized and investigated. IVM of early follicle stages from which mature fertilizable oocytes can be retrieved is one way to avoid the risk of transmitting malignant cells when re-implanting frozen-thawed ovarian tissue. This has been achieved in mice [116], and a metaphase II oocyte was retrieved from a primate follicle cultured from a pre-antral follicle [117]. In humans, long-term culture of preantral follicles to early antral stages has been achieved [84, 118]; however, research is still required to establish this as a possible clinical application. Another approach suggests the transfer of isolated human primordial follicles into an artificial ovary—a specially created scaffold—so as to provide an alternative way of restoring fertility in patients who cannot benefit from transplantation of cryopreserved ovarian tissue [119, 120].

References

1. Stoop D, Maes E, Polyzos NP, et al. Does oocyte banking for anticipated gamete exhaustion influence future relational and reproductive choices? A follow up of bankers and non-bankers. *Hum Reprod*. 2015;30:338–44.
2. Andersen CY, Kristensen SG. Novel use of the ovarian follicular pool to postpone menopause and delay osteoporosis. *Reprod Biomed Online*. 2015;31:128–31.
3. Jemal A, Siegel R, Ward E, et al. Cancer statistics. *CA Cancer J Clin*. 2007;57:43–66.
4. Meirrow D, Hardan I, Dor J, et al. Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. *Hum Reprod*. 2008;23:1007–13.
5. Von Wolff M, Donnez J, Hovatta O, et al. Cryopreservation and autotransplantation of human ovarian tissue prior to cytotoxic therapy—A technique in its infancy but already successful in fertility preservation. *Eur J Cancer*. 2009;45:1547–53.
6. El Issaoui M, Giorgione V, Mamsen LS, Rechnitzer C, Birkebæk N, Clausen N, Kelsey TW, Andersen CY. Effect of first line cancer treatment on the ovarian reserve and follicular density in girls under the age of 18 years. *Fertil Steril*. 2016;106:1757–62.

7. Lamar CA, Decherney AH. Fertility preservation: State of the science and future research directions. *Fertil Steril.* 2008;91:316–19.
8. Donnez J, Dolmans MM, Diaz C, Pellicer A. Ovarian cortex transplantation: Time to move on from experimental studies to open clinical application. *Fertil Steril.* 2015;104:1097–8.
9. Meirow D, Biederman H, Anderson RA, et al. Toxicity of chemotherapy and radiation on female reproduction. *Clin Obstet Gynecol.* 2010;53:727–39.
10. Kalich-Philosoph L, Roness H, Carmely A, et al. Cyclophosphamide triggers follicle activation and “burnout”; AS101 prevents follicle loss and preserves fertility. *Sci Transl Med.* 2013;5:185ra62.
11. Nicholson HS, Byrne J. Fertility and pregnancy after treatment for cancer during childhood or adolescence. *Cancer.* 1993;71:3392–9.
12. Meirow D. Reproduction post-chemotherapy in young cancer patients. *Mol Cell Endocrinol.* 2000;169:123–31.
13. Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. *Cancer.* 2007;110:2222–9.
14. Meirow D, Lewis H, Nugent D, et al. Subclinical depletion of primordial follicular reserve in mice treated with cyclophosphamide: Clinical importance and proposed accurate investigative tool. *Hum Reprod.* 1999;14:1903–7.
15. Wallace WHB, Thomson AB, Saran F, Kelsey TW. Predicting age of ovarian failure after radiation to a field that includes the ovaries. *Int J Radiat Oncol Biol Phys.* 2005;62:738–44.
16. Lushbaugh CC, Casarett GW. The effects of gonadal irradiation in clinical radiation therapy: A review. *Cancer.* 1976;37:1111–20.
17. Donnez J, Dolmans MM. Fertility preservation in women. *Nat Rev Endocrinol.* 2013;9:735–49.
18. Lambertini M, Del Mastro L, Pescio MC, et al. Cancer and fertility preservation: International recommendations from an expert meeting. *BMC Med.* 2016;14:1.
19. Wallace WH, Smith AG, Kelsey TW, et al. Fertility preservation for girls and young women with cancer: Population-based validation of criteria for ovarian tissue cryopreservation. *Lancet Oncol.* 2014;15:1129–36.
20. Schmidt KT, Larsen EC, Andersen CY, Andersen AN. Risk of ovarian failure and fertility preserving methods in girls and adolescents with a malignant disease. *BJOG.* 2010;117:163–74.
21. Badawy A, Elnashar A, El-Ashry M, Shahat M. Gonadotropin-releasing hormone agonists for prevention of chemotherapy-induced ovarian damage: Prospective randomized study. *Fertil Steril.* 2009;91:694–7.
22. Peeyra Pacheco B, Méndez Ribas JM, Milone G, et al. Use of GnRH analogs for functional protection of the ovary and preservation of fertility during cancer treatment in adolescents: A preliminary report. *Gynecol Oncol.* 2001;81:391–7.
23. Blumenfeld Z, von Wolff M. GnRH-analogues and oral contraceptives for fertility preservation in women during chemotherapy. *Hum Reprod Update.* 2008;14:543–52.
24. Munhoz RR, Pereira AA, Sasse AD, et al. Gonadotropin releasing hormone agonists for ovarian function preservation in premenopausal women undergoing chemotherapy for early-stage breast cancer: A systematic review and meta-analysis. *JAMA Oncol.* 2016;2:65–73.
25. Dolmans MM, Hollanders de Ouderaen S, Demylle D, et al. Utilization rates and results of long-term embryo cryopreservation before gonadotoxic treatment. *J Assist Reprod Genet.* 2015;32:1233–7.
26. Donnez J, Dolmans MM. Ovarian tissue freezing: Current status. *Curr Opin Obstet Gynecol.* 2015;27:222–30.
27. von Wolff M, Thaler CJ, Frambach T, et al. Ovarian stimulation to cryopreserve fertilized oocytes in cancer patients can be started in the luteal phase. *Fertil Steril.* 2008;92:1360–5.
28. Cakmak H, Katz A, Cedars MI, et al. Effective method for emergency fertility preservation: Random-start controlled ovarian stimulation. *Fertil Steril.* 2013;100:1673–80.
29. Andersen CY, Rosendahl M, Byskov AG, et al. Two successful pregnancies following autotransplantation of frozen/thawed ovarian tissue. *Hum Reprod.* 2008;23:2266–72.
30. Rosendahl M, Schmidt KT, Ernst E, et al. Cryopreservation of ovarian tissue for a decade in Denmark: A view of the technique. *Reprod Biomed Online.* 2011;22:162–71.
31. Donnez J, Dolmans MM, Pellicer A, et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: A review of 60 cases of reimplantation. *Fertil Steril.* 2013;99:1503–13.
32. Wilken-Jensen HN, Kristensen SG, Jeppesen JV, et al. Developmental competence of oocytes isolated from surplus medulla tissue in connection with cryopreservation of ovarian tissue for fertility preservation. *Acta Obstet Gynecol Scand.* 2014;93:32–7.
33. Yin H, Jiang H, Kristensen SG, et al. Vitrification of *in vitro* matured oocytes collected from surplus ovarian medulla tissue resulting from fertility preservation of ovarian cortex tissue. *J Assist Reprod Genet.* 2016;33:741–6.
34. Fasano G, Moffa F, Dechène J, et al. Vitrification of *in vitro* matured oocytes collected from antral follicles at the time of ovarian tissue cryopreservation. *Reprod Biol Endocrinol.* 2011;9:150.
35. Prasath EB, Chan ML, Wong WH, Lim CJ, Tharmalingam MD, Hendricks M, Loh SF, Chia YN. First pregnancy and live birth resulting from cryopreserved embryos obtained from *in vitro* matured oocytes after oophorectomy in an ovarian cancer patient. *Hum Reprod.* 2014;29:276–8.
36. Segers I, Mateizel I, Van Moer E, et al. *In vitro* maturation (IVM) of oocytes recovered from ovariectomy specimens in the laboratory: A promising “*ex vivo*” method of oocyte cryopreservation resulting in the first report of an ongoing pregnancy in Europe. *J Assist Reprod Genet.* 2015;32:1221–31.
37. Macklon KT, Jensen AK, Loft A, et al. Treatment history and outcome of 24 deliveries worldwide after autotransplantation of cryopreserved ovarian tissue, including two new Danish deliveries years after autotransplantation. *J Assist Reprod Genet.* 2014;31:1557–64.
38. Jensen AK, Kristensen SG, Macklon KT, et al. Outcomes of transplants of cryopreserved ovarian tissue to 41 women in Denmark. *Hum Reprod.* 2015;30:2838–45.
39. Jensen AK, Macklon KT, Fedder J, Ernst E, Humaidan P, Andersen CY. 86 successful births and 9 ongoing pregnancies worldwide in women transplanted with frozen-thawed ovarian tissue: Focus on birth and perinatal outcome in 40 of these children. *J Assist Reprod Genet.* 2017;34:325–6.
40. Greve T, Ernst E, Markholt S, Schmidt KT, Andersen CY. Legal termination of a pregnancy resulting from transplanted cryopreserved ovarian tissue. *Acta Obstet Gynecol Scand.* 2010;89:1589–91.
41. Cooper GS, Thorp JM Jr. FSH levels in relation to hysterectomy and to unilateral oophorectomy. *Obstet Gynecol.* 1999;94:969–72.
42. Lass A. The fertility potential of women with a single ovary. *Hum Reprod Update.* 1999;5:546–50.
43. Anderson RA, Wallace WH. Fertility preservation in girls and young women. *Clin Endocrinol (Oxf).* 2011;75:409–19.
44. Hovatta O, Silye R, Krausz T, et al. Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants. *Hum Reprod.* 1996;11:1268–72.
45. Fuller B, Paynter S. Fundamentals of cryobiology in reproductive medicine. *Reprod Biomed Online.* 2004;9:680–91.
46. Donnez J, Dolmans MM, Demylle D, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet.* 2004;364:1405–10.
47. Kawamura K, Cheng Y, Suzuki N, et al. Hippo signaling disruption and akt stimulation of ovarian follicles for infertility treatment. *Proc Natl Acad Sci USA.* 2013;110:17474–9.
48. Suzuki N, Yoshioka N, Takae S, et al. Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency. *Hum Reprod.* 2015;30:608–15.

49. Keros V, Xella S, Hultenby K, et al. Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. *Hum Reprod.* 2009;24:1670–83.
50. Amorim CA, Curaba M, Van Langendonck A, Dolmans MM, Donnez J. Vitrification as an alternative means of cryopreserving ovarian tissue. *Reprod Biomed Online.* 2011;23: 160–86.
51. Ting AY, Yeoman RR, Lawson MS, Zelinski MB. *In vitro* development of secondary follicles from cryopreserved rhesus macaque ovarian tissue after slow rate freeze or vitrification. *Hum Reprod.* 2011;26:2461–72.
52. Isachenko V, Isachenko E, Weiss JM, Todorov P, Kreienberg R. Cryobanking of human ovarian tissue for anti-cancer treatment: Comparison of vitrification and conventional freezing. *Cryo Lett.* 2009;30:449–54.
53. Oktem O, Alper E, Balaban B, et al. Vitrified human ovaries have fewer primordial follicles and produce less antimüllerian hormone than slow-frozen ovaries. *Fertil Steril.* 2011;95:2661–4.e1.
54. Martinez-Madrid B, Camboni A, Dolmans MM, et al. Apoptosis and ultrastructural assessment after cryopreservation of whole human ovaries with their vascular pedicle. *Fertil Steril.* 2007;87:1153–65.
55. Bromer JG, Patrizio P. Fertility preservation: The rationale for cryopreservation of the whole ovary. *Semin Reprod Med.* 2009;27:465–71.
56. Schmidt KL, Ernst E, Byskov AG, Andersen N, Andersen A. Survival of primordial follicles following prolonged transportation of ovarian tissue prior to cryopreservation. *Hum Reprod.* 2003;18:2654–9.
57. Dittrich R, Hackl J, Lotz L, et al. Pregnancies and live births after 20 transplantations of cryopreserved ovarian tissue in a single center. *Fertil Steril.* 2015;103:462–8.
58. Rosendahl M, Andersen CY, Ernst E, et al. Ovarian function after removal of an entire ovary for cryopreservation of pieces of cortex prior to gonadotoxic treatment: A follow-up study. *Hum Reprod.* 2008;23:2475–83.
59. Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG. Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at -196 °C. *Endocrinology.* 1999;140:462–71.
60. Schmidt KT, Rosendahl M, Ernst E, et al. Autotransplantation of cryopreserved ovarian tissue in 12 women with chemotherapy-induced premature ovarian failure: The Danish experience. *Fertil Steril.* 2011;95:695–701.
61. Stern CJ, Gook D, Hale LG, et al. First reported clinical pregnancy following heterotopic grafting of cryopreserved ovarian tissue in a woman after a bilateral oophorectomy. *Hum Reprod.* 2013;28:2996–9.
62. Kristensen SG, Giorgione V, Humaidan P, Alsberg B, Bjørn AB, Ernst E, Andersen CY. Fertility preservation and refreezing of transplanted ovarian tissue—a potential new way of managing patients with low risk of malignant cell recurrence. *Fertil Steril.* 2017;107(5):1206–1213.
63. Schmidt KL, Andersen CY, Loft A, et al. Follow-up of ovarian function post-chemotherapy following ovarian cryopreservation and transplantation. *Hum Reprod.* 2005;20:3539–46.
64. Donnez J, Silber S, Andersen CY, et al. Children born after autotransplantation of cryopreserved ovarian tissue. A review of 13 live births. *Ann Med.* 2011;43:437–50.
65. Hornshøj VG, Dueholm M, Mamsen LS, Ernst E, Andersen CY. Hormonal response in patients transplanted with cryopreserved ovarian tissue is independent of whether freezing was performed in childhood or adulthood. *J Assist Reprod Genet.* 2021;38:3039–45.
66. Poirot C, Abirached F, Prades M, et al. Induction of puberty by autograft of cryopreserved ovarian tissue. *Lancet.* 2012;379: 588.
67. Ernst E, Kjærsgaard M, Birkebæk NH, et al. Case report: Stimulation of puberty in a girl with chemoand radiation therapy induced ovarian failure by transplantation of a small part of her frozen/thawed ovarian tissue. *Eur J Cancer.* 2013;49:911–4.
68. Donnez J, Dolmans MM. Ovarian cortex transplantation: 60 reported live births brings the success and worldwide expansion of the technique towards routine clinical practice. *J Assist Reprod Genet.* 2015;32:1167–70.
69. Gellert SE, Pors SE, Kristensen SG, Bay-Bjørn AM, Ernst E, Yding Andersen C. Transplantation of frozen-thawed ovarian tissue: An update on worldwide activity published in Peer-reviewed papers And on the Danish cohort. *J Assist Reprod Genet.* 2018;35:561–70.
70. Donnez J, Dolmans MM. Fertility preservation in women. *N Engl J Med.* 2017;377:1657–1665.
71. Andersen CY. Success and challenges in fertility preservation after ovarian tissue grafting. *Lancet.* 2015;385:1947–8.
72. Dolmans MM, von Wolff M, Poirot C, Diaz-Garcia C, Cacciottola L, Boissel N, Liebenthron J, Pellicer A, Donnez J, Andersen CY. Transplantation of cryopreserved ovarian tissue in A series of 285 women: A review of five leading European centers. *Fertil Steril.* 2021;115:1102–15.
73. Dolmans MM, Donnez J, Camboni A, et al. IVF outcome in patients with orthotopically transplanted ovarian tissue. *Hum Reprod.* 2009;24:2778–87.
74. Meirow D, Levron J, Eldar-Geva T, et al. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. *N Engl J Med.* 2005;353:318–21.
75. Greve T, Schmidt KT, Kristensen SG, et al. Evaluation of the ovarian reserve in women transplanted with frozen and thawed ovarian cortical tissue. *Fertil Steril.* 2012;97:1394–8.e1.
76. Andersen CY, Silber SJ, Bergholdt SH, Jorgensen JS, Ernst E. Long-term duration of function of ovarian tissue transplants: Case reports. *Reprod Biomed Online.* 2012;25:128–32.
77. Rauff S, Giorgione V, Yding Andersen C. Potential malignant cell contamination in transplanted ovarian tissue. *Expert Opin Biol Ther.* 2016;16:285–9.
78. Dolmans MM, Luyckx V, Donnez J, et al. Risk of transferring malignant cells with transplanted frozen-thawed ovarian tissue. *Fertil Steril.* 2013;99:1514–22.
79. Bastings L, Beerendonk CC, Westphal JR, et al. Autotransplantation of cryopreserved ovarian tissue in cancer survivors and the risk of reintroducing malignancy: A systematic review. *Hum Reprod Updat.* 2013;19:483–506.
80. Rosendahl M, Greve T, Andersen CY. The safety of transplanting cryopreserved ovarian tissue in cancer patients: A review of the literature. *J Assist Reprod Genet.* 2013;30:11–24.
81. Dolmans MM, Marinescu C, Saussoy P, et al. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood.* 2010;116:2908–14.
82. Greve T, Clasen-Linde E, Andersen MT, et al. Cryopreserved ovarian cortex from patients with leukemia in complete remission contains no apparent viable malignant cells. *Blood.* 2012;120:4311–6.
83. Dolmans MM, Yuan WY, Camboni A, et al. Development of antral follicles after xenografting of isolated small human preantral follicles. *Reprod Biomed Online.* 2008;16:705–11.
84. Yin H, Kristensen SG, Jiang H, et al. Survival and growth of isolated pre-antral follicles from human ovarian medulla tissue during long-term 3D culture. *Hum Reprod.* 2016;31:1531–9.
85. Stern CJ, Gook D, Hale LG, et al. Delivery of twins following heterotopic grafting of frozen-thawed ovarian tissue. *Hum Reprod.* 2014;29(8):1828.
86. van der Steeg JW, Steures P, Eijkemans MJ, et al. Pregnancy is predictable: A large-scale prospective external validation of the prediction of spontaneous pregnancy in subfertile couples. *Hum Reprod.* 2007;22:536–42.
87. Jahnukainen K, Ehmcke J, Hou M, Schlatt S. Testicular function and fertility preservation in male cancer patients. *Best Pract Res Clin Endocrinol Metab.* 2011;252:287–302.

88. Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: Who is at risk and what can be offered? *Lancet Oncol.* 2005;6:209–18.
89. Aubier F, Flamant F, Brauner R, et al. Male gonadal function after chemotherapy for solid tumors in childhood. *J Clin Oncol.* 1989;7:304–9.
90. Siimes MA, Rautonen J. Small testicles with impaired production of sperm in adult male survivors of childhood malignancies. *Cancer.* 1990;65:1303–6.
91. Ridola V, Fawaz O, Aubier F, et al. Testicular function of survivors of childhood cancer: A comparative study between ifosfamide- and cyclophosphamidebased regimens. *Eur J Cancer.* 2009;45:814–8.
92. Meistrich ML, Wilson G, Brown BW, et al. Impact of cyclophosphamide on longterm reduction in sperm count in men treated with combination chemotherapy for ewing and soft tissue sarcomas. *Cancer.* 1992;70:2703–12.
93. Rivkees SA, Crawford JD. The relationship of gonadal activity and chemotherapy-induced gonadal damage. *JAMA.* 1988;259:2123–5.
94. Jahnukainen K, Ehmcke J, Schlatt S. Testicular xenografts: A novel approach to study cytotoxic damage in juvenile primate testis. *Cancer Res.* 2006;66:3813–8.
95. Jahnukainen K, Ehmcke J, Nurmi M, et al. Irradiation causes acute and long-term spermatogonial depletion in cultured and xenotransplanted testicular tissue from juvenile nonhuman primates. *Endocrinology.* 2007;148:5541–8.
96. Rowley MJ, Leach DR, Warner GA, Heller CG. Effect of graded doses of ionizing radiation on the human testis. *Radiat Res.* 1974;59:665–78.
97. Centola GM, Keller JW, Henzler M, Rubin P. Effect of low-dose testicular irradiation on sperm count and fertility in patients with testicular seminoma. *J Androl.* 1994;15:608–13.
98. van Alphen MMA, van den Kant HJG, de Rooij DG. Repopulation of the seminiferous epithelium of the rhesus monkey after X irradiation. *Radiat Res.* 1988;113:487–500.
99. Jensen CFS, Dong L, Gul M, Fode M, Hildorf S, Thorup J, Hoffmann E, Cortes D, Fedder J, Andersen CY, Sønksen J. Fertility preservation in boys facing gonadotoxic cancer therapy. *Nat Rev Urol.* 2022;19:71–83.
100. Kamischke A, Jurgens H, Hertle L, et al. Cryopreservation of sperm from adolescents and adults with malignancies. *J Androl.* 2004;25:586–92.
101. Baukloh V, German Society for Human Reproductive Biology. Retrospective multicentre study on mechanical and enzymatic preparation of fresh and cryopreserved testicular biopsies. *Hum Reprod.* 2002;17:1788–94.
102. Meseguer M, Garrido N, Remohi J, et al. Testicular sperm extraction (TESE) and ICSI in patients with permanent azoospermia after chemotherapy. *Hum Reprod.* 2003;18:1281–5.
103. Picton HM, Wyns C, Anderson RA, ESHRE Task Force On Fertility Preservation In Severe Diseases, et al. A European perspective on testicular tissue cryopreservation for fertility preservation in pre-pubertal and adolescent boys. *Hum Reprod.* 2015;30:2463–75.
104. Brinster RL. Male germline stem cells: From mice to men. *Science.* 2007;316:404–5.
105. Dores C, Alpaugh W, Dobrinski I. From *in vitro* culture to *in vivo* models to study testis development and spermatogenesis. *Cell Tissue Res.* 2012;349:691–702.
106. Brook PF, Radford JA, Shalet SM, et al. Isolation of germ cells from human testicular tissue for low temperature storage and auto-transplantation. *Fertil Steril.* 2001;75:269–74.
107. Keros V, Rosenlund B, Hultenby K, et al. Optimizing cryopreservation of human testicular tissue: Comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. *Hum Reprod.* 2005;20:1676–87.
108. Kvist K, Thorup J, Byskov AG, et al. Cryopreservation of intact testicular tissue from boys with cryptorchidism. *Hum Reprod.* 2006;21:484–91.
109. Schlatt S, Kim SS, Gosden R. Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. *Reproduction.* 2002;124:339–46.
110. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA.* 1994;91:11298–302.
111. Schlatt S, Rosiepen G, Weinbauer GF, et al. Germ cell transfer into rat, bovine, monkey and human testes. *Hum Reprod.* 1999;14:144–50.
112. Sadri-Ardekani H, Mizrak SC, van Daalen SK, et al. Propagation of human spermatogonial stem cells *in vitro*. *JAMA.* 2009;302:2127–34.
113. Sadri-Ardekani H, Akhondi MA, van der Veen F, et al. *In vitro* propagation of human prepubertal spermatogonial stem cells. *JAMA.* 2011;305:2416–8.
114. Hermann BP, Sukhwani M, Winkler F, et al. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. *Cell Stem Cell.* 2012;11:715–26.
115. Struijk RB, Mulder CL, van der Veen F, et al. Restoring fertility in sterile childhood cancer survivors by autotransplanting spermatogonial stem cells: Are we there yet? *Biomed Res Int.* 2013;2013:903142.
116. Xu M, Kreeger PK, Shea LD, Woodruff TK. Tissue engineered follicles produce live, fertile offspring. *Tissue Eng.* 2006;12:2739–46.
117. Xu J, Lawson MS, Yeoman RR, et al. Secondary follicle growth and oocyte maturation during encapsulated three-dimensional culture in rhesus monkeys: Effects of gonadotrophins, oxygen and fetauin. *Hum Reprod.* 2011;26:1061–72.
118. Xiao S, Zhang J, Romero MM, Smith KN, Shea LD, Woodruff TK. *In vitro* follicle growth supports human oocyte meiotic maturation. *Sci Rep.* 2015 Nov 27;5:17323.
119. Luyckx V, Dolmans MM, Vanacker J, et al. A new step toward the artificial ovary: Survival and proliferation of isolated murine follicles after autologous transplantation in a fibrin scaffold. *Fertil Steril.* 2014;101:1149–56.
120. Laronda MM, Rutz AL, Xiao S, Whelan KA, Duncan FE, Roth EW, Woodruff TK, Shah RN. A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nat Commun.* 2017 May 16;8:15261.

UTERUS TRANSPLANTATION*In Transition from Experimental to Clinical Procedure***Mats Brännström, Ghada Hussein, Ali Khatibi, and Pernilla Dahm-Kähler**

Introduction

Uterus transplantation (UTx) during the last decades has developed as a novel infertility treatment for absolute uterine factor infertility (AUFI) caused by absence of a functional uterus. After systematic animal research [1] over a decade, and involving rodents, domestic species, and non-human primates, the first clinical UTx trial was launched in 2013 [2]. This was a live donor (LD) UTx trial and one out of nine participating women gave birth to the world's first UTx baby in September 2014 [3]. Since then, more than 10 clinical UTx trials have been initiated, with a mix between LD UTx and deceased donor (DD) UTx. Based on data from all registered ongoing trials and our personal experience, we describe plausible patient groups for UTx and cover different techniques of surgery and assisted reproduction in conjunction with UTx.

Plausible uterus transplantation patients

The diagnosis of AUFI is based on uterine absence (surgical/congenital) or a uterine defect (anatomic/functional), and the prevalence of AUFI is approximately 20,000 women of childbearing age in a total population of 100 million [4]. Women with a dysfunctional uterus should at first, before classified as AUFI, be considered to have a relative uterine infertility, since there may exist a chance for a pregnancy leading to live birth, either spontaneously or after medical intervention, such as IVF.

Hysterectomy at fertile age is the most prevalent cause of anatomical uterine absence, and only in the United States around 150,000 hysterectomies are performed each year in fertile-aged women [5]. One malignancy-associated cause of hysterectomy is cervical cancer, with around 30% of cervical cancer patients being diagnosed during fertile age [6]. Fertility-sparing surgery by trachelectomy can be applied in selective cases with smaller tumours according to international guidelines. Uterus transplantation would only be recommended for patients undergoing radical hysterectomy solely and not in patients also receiving adjuvant radiation treatment. Other uterine malignancies, such as leiomyosarcoma, endometrial stromal sarcoma, and endometrial cancer, are very uncommon during the reproductive years [7] but occur occasionally. At least five years should pass since malignancy diagnosis until considering UTx, so that the risk of recurrence of malignancy is minimal, taking into account that a UTx patient is immunosuppressed. A less common cause of iatrogenic AUFI in young women is hysterectomy in conjunction with parturition, occurring in around 5 in 10,000 deliveries [8], with caesarean delivery being an independent risk factor for this so-called peripartum emergency hysterectomy [9]. Massive or inoperable leiomyoma may have a significant negative impact on quality of life, and

hysterectomy is the only available treatment. Around 2.5% of women under the age of 40 years have undergone hysterectomy, specifically due to leiomyoma [10].

The Mayer–Rokitansky–Küster–Hauser syndrome (MRKHs), a condition with uterine aplasia in a female with normal karyotype and normal secondary sex characteristics, is seen in around 1 in 4500 females [11]. So far, a great majority of recipients in UTx procedures worldwide have been women with MRKHs.

A number of uterine factor infertility conditions exist in the presence of a uterus and they may initially be classified as relative uterine factor infertility, until the potential for pregnancy and live birth have been fully ruled out. The prevalence of myoma is around 25% in a normal IVF population, with submucous and large intramural leiomyomas being associated with decreased rates of implantation and pregnancy [12]. Myomectomy is the surgery of choice and is effective in many instances. Thus, repeated pregnancy failures after myomectomy, where also multiple rounds of IVF have been applied, could make a patient eligible for hysterectomy and UTx.

Presence of intrauterine adhesions (IUA), also named Asherman's syndrome, has a prevalence of around 1.5% among women of fertile age [13]. The condition is usually secondary to endometritis or postpartum surgical curettage. Hysteroscopic removal of adhesions can restore infertility in mild, moderate, and severe IUA in around 90%, 70%, and 30% of cases, respectively [14].

Radiotherapy, as local pelvic irradiation or total body irradiation, causes considerable reduction of uterine volume, with implantation failure or increased rates of miscarriage and late pregnancy loss seen in a majority of cases [15]. In the event that a pregnancy progresses into the third trimester, adverse perinatal outcome is seen in a majority of cases [16]. In most women receiving radiation therapy over the pelvic region, the treatment will be toxic to ovaries, and oocyte donation may be needed.

Congenital uterine malformations are caused by disturbances during fetal life in the formation, development, or fusion of the Müllerian (paramesonephric) ducts. The prevalence of uterine malformations in the general population is around 5%–7% and a majority of these malformations are not associated with infertility, but in women with recurrent spontaneous abortion the rate of partial uterine malformations is around 15% [17]. The MRKHs (described earlier) is the most severe congenital uterine malformation, with the existence of only a midline rudiment and lateral uterine tissue buds [11].

The most prevalent uterine malformation is the septate uterus, accounting for around 30% of all uterine malformations [17]. In women with untreated septate uteri the rate of spontaneous abortion is around 75% [18] and after hysteroscopic septate resection the miscarriage rate is in the same range as in women with normal uteri. The bicornuate uterus is the second most common partial uterine malformation, representing around 25% of uterine

malformations [17]. In these women, the rate of spontaneous abortion is around 35%, and metroplasty surgery has a questionable effect [18].

Unicornuate uterus and uterus didelphys are associated with undersized uterine cavities because of only unilateral development of the Müllerian ducts (unicornuate uterus) or an absence of fusion of the Müllerian ducts (didelphys), and together they represent around 20% of all uterine malformations [17]. They are associated with increased miscarriage rate (30%) and decreased (50%) live birth rate, and there is no effective surgical method to improve fertility results [18].

The hypoplastic uterus and the T-shaped uterus are two infrequent forms of uterine malformation, with live birth rates of less than 10% [17, 18].

Types of uterus transplantation

Uterus transplantation can be either from a LD or a deceased donor (DD), also called a multi-organ donor. The obvious advantages of DD UTx, as compared to LD UTx, are that the donor is not exposed to any surgical risk, easier surgery since the distal ureters can be transected proximal and distal to the parametrium, and good access to long vascular pedicles with large-diameter vessels as end vessel segments, to be used for anastomosis. The advantages of LD UTx are that a full medical history is available, the surgery can be planned well ahead with a prepared multidisciplinary, and the cold ischemia time can be kept short.

Deceased donor hysterectomy is always performed by laparotomy and LD hysterectomy can be done by either laparotomy or robotic-assisted laparoscopy. Transplantation into the recipient can be done by either laparotomy or by robotics. The different surgical scenarios are described below.

Deceased donor hysterectomy—open surgery technique

In DD hysterectomy, laparotomy, also referred to as open surgery, is part of multi-organ recovery. Thus, the quality of the life-saving abdominal and thoracic organs to be transplanted has to be fully considered in relation to uterus procurement. There is a clear advantage to performing the dissection of uterine blood vessels, including the internal iliac vessels, as well as ligations of branches prior to retrieval of vital organs so that the team in due time will recover an optimal uterus graft. This early uterine preparation, before procurement of other organs, may be estimated to take around 1–2 h, and the major steps are described as follows.

Importantly, an initial vaginal cleansing, including antifungal vaginal pessary if suspicion of fungal infection exists, should be performed. The preferred abdominal incision is a complete midline incision with the possibility to add bilateral inguinal incisions to acquire full access to the pelvic sidewalls. The common iliac arteries are identified, and dissection is then directed distally towards the branching of uterine arteries, with dissections to a distance of around 2 cm from the uterine artery crossings over the ureters. The ureters are ligated at this position, transected and tagged since they mark the uterine artery location. Then the round ligaments are transected bilaterally, and a large bladder peritoneum flap on the uterine side is prepared. The ureters are identified close to their inlet into the bladder and transected. The obliterated umbilical vessels are identified, ligated around 2 cm from their branching from uterine arteries, and tagged. The tag sutures will later be used for traction to expose to the

deep internal iliac vessels and in particular the veins. The internal iliac arteries and veins are harvested with dissections from the bifurcations between the internal and external iliac vessels, with ligations and transections of all major branches. These arterial branches include the gluteal, obturator, and inferior rectal arteries. The branches of the internal iliac vein show great interindividual variations and cautious dissection of the thin-walled veins is advised. Thereafter, the dissection is directed towards the parametrial mass, with the uterine artery and uterine veins centrally in this mass. Venous branches are divided between clips, which are secured by sutures, since there is a high risk that classical ligatures may slip and render an opening of the branch. After completion of the bilateral vessel dissections, the surgery is focused on the rectovaginal space with transection of the uterosacral ligaments, leaving good lengths on the uterine side for later uterine fixation in the recipient. After this procedure, the external iliac arteries are exposed for the planned catheterization and flushing of the uterus.

The abdominal and thoracic organ procurement teams may then join and start the procurement of the vital organs and prepare for flushing. Separate uterine flushing, by arterial catheters inside the external iliac arteries cranially to the inguinal ligaments with clamping of the aorta above the bifurcation, is performed to accomplish an effective flush of the uterus. After completed procurement of thoracic and abdominal organs, with the uterus being continuously flushed and cooled during this procedure, the final uterus procurement may be performed. The vagina is transected at a level around 2–3 cm from the fornix and the internal iliac vessels are clamped and transected at a level which will provide optimal segments for anastomosis. The uterus is removed and transferred to the back table.

Live donor hysterectomy—open surgery technique

A laparotomy by a classic sub-umbilical, midline incision is the technique that has been used in the majority of LD hysterectomies performed so far. After entering the abdomen, the round ligaments are divided bilaterally at lateral positions. A large peritoneal bladder flap, attached to the uterine graft, is dissected by monopolar diathermia. The peritoneal bladder flap will be an essential part of fixation of the grafted uterus in the recipient and will also cover the vesico-uterine fossa, thereby minimizing risk of intestinal herniation at this site.

Bilateral dissections of the pelvic sidewall are performed as described herein for unilateral dissection. The obliterated umbilical artery is identified close to the inguinal ligament and then ligated and divided around 2 cm from the bifurcation of the uterine artery. Traction in this arterial stump will aid in further dissection, and the position of the umbilical artery will also guide in safe dissection on the lateral side, in order not to induce any damage to the uterine artery or veins. The ureter is mobilized from the level of the iliac crossing and dissected in a distal direction towards the ureteric tunnel. The dissection should be performed from a cranial angle and with great caution to avoid any injury to the deep uterine veins, which in a majority of cases go beneath the ureter. Several small arterial and venous branches, especially close to and in the ureteric tunnel, are coagulated or ligated before severance. Bipolar, rather than monopolar, diathermy should be used in order to avoid heat transmission to the ureter. When the ureter is relatively free for the length of the ureteric tunnel, and only covered by the uterine artery and any overriding uterine veins as well as not attached to paracervical tissue inside the tunnel, the ureter may be identified in front of the uterine

artery towards the bladder. Identification is aided by intermittent ureteric traction cranially to the ureteric tunnel. Multiple vein plexuses are attached to the ureter at its course from the ureteric tunnel towards the inlet into the bladder. These need to be delicately dissected away from the ureter. Notably, the uterine veins may be directed in a knee towards the bladder, before arching down towards the pelvic sidewall and the inlet into the internal iliac vein.

The arterial segment between the bifurcation of the posterior branch of the internal iliac artery and the uterine artery at the cervix are kept attached to the graft to act as the arterial conduit at transplantation. Several branches from the internal iliac artery, such as iliolumbar, lateral sacral, pudendal, middle rectal, vaginal, and obturator branch, are ligated and divided. The umbilical artery has already been transected (see above). One major trunk of the arterial supply of the anterior branch of the internal iliac artery and the continuation into the uterine artery will be accomplished on each side of the graft. Importantly, the major posterior branch of the internal iliac artery, the gluteal artery, should be kept not to compromise blood flow to the gluteal muscle.

The most difficult part of the open LD hysterectomy is the dissection and procurement of the uterine veins, where the goal is to procure one to two large veins, with a segment of the internal iliac vein on each side. The uterine veins may ride over or under the ureter, with also anatomical side-differences within patients. This dissection involves severance of major uterine vein branches, preferably divided by clips, which are secured by sutures to avoid gliding of the suture. The deep dissection of the distal part of the uterine veins begins on the pelvic sidewall, in order to identify the internal iliac vein with all its branches, and dissection is then towards the uterus. The venous vascular pedicle should optimally, at its end, comprise a segment of the internal iliac vein, in order to get a vein with a well-defined wall that will facilitate the vascular anastomosis surgery. In cases where there are two major uterine veins on one side and with alternate passage in relation to the ureter, one vein has to be divided before removing the graft and then repaired by end-to-end anastomosis on the back table. Additionally, the uterine branch of the utero-ovarian vein is dissected before the entry of the ovarian vein in order to be used as extra venous outflow, if necessary. Salpingectomy and transection of the utero-ovarian ligament is performed during this dissection. At this stage, when full pelvic sidewall dissection has been completed bilaterally, the surgery is directed towards the posterior aspect of the uterus with the initial step being opening of the rectovaginal space.

The utero-sacral ligaments are then divided and the paravaginal tissue is dissected to identify and divide the vaginal arteries and veins. Division of the vagina is performed by scissors and/or bipolar diathermy with a vaginal rim on the uterine side of around 2 cm in order to facilitate the vaginal–vaginal anastomosis in the recipient (see below). The uterus is at this stage only attached by the bilateral arterial and venous vascular pedicles. Vascular clamps are placed in the following order: first on the internal iliac arteries just distal to the branching of the gluteal arteries, thereafter on the internal iliac veins to acquire a segment, and at last distally on the uterine branch of the utero-ovarian vein. The vessels are divided on the uterine side of the vascular clamps and cut sharp in order to make the vessel walls optimal for anastomosis suturing. The uterus is quickly moved to the back table for immediate chilling and flushing. The division sites on the major arteries and veins are closed by continuous sutures, and ovary-pexy is performed to the pelvic sidewalls, laterally to the common iliacs.

Surgery ends by standard technique, including haemostatic control, mass suture, subcutaneous suture, and intracutaneous skin suture.

Live donor hysterectomy—robotics technique

A robotic system with a four-arm set-up and dual console is recommended. Two robotic surgeons and one laparoscopic surgeon carry out surgery. The donor is placed in steep (angle around 28°) Trendelenburg position and with enabled side-docking (45°) towards the patient's hip, in order to gain easy access for a non-invasive uterine manipulator, which will be used to alter the position of the uterus during the different steps of surgery. A robotic optic scope of 0° usually provides advantageous vision, but in narrow spaces on the pelvic sidewall it may be changed to one with a 30° angle. The instruments to be used include a Maryland bipolar forceps, monopolar curved scissors, a large needle driver, a clip applier for medium and large clips, a prograsp forceps, and a vessel sealer.

The surgery starts with transection of the round ligaments and dissection of the large peritoneal bladder flap, as also described for open technique for LD hysterectomy (see above). The pelvic side wall dissection should be performed on one side at a time to make the robotic surgery efficient and less time-consuming. The right pelvic side is preferably the initial side due to the ergonomics of the assistant and accessibility. Initially, the ureter is dissected between the crossing over the iliac vessel and until around 5 mm before it reaches the ureteric tunnel. This tunnel is defined as the anatomical space where the uterine artery overrides the ureter, which is attached to the uterine cervix. Then the dissection of ureteric tunnel, with the aim to free the ureter from any attachment, starts from the cranial side of the tunnel. The surgery involves meticulous and precise dissection, in order to separate the ureter from its attachments without injuring the surrounding uterine artery and uterine veins. The ureter should be completely mobilized by this dissection from a proximal angle to a level near a centimetre caudally to the uterine artery crossing. Several small arteries and veins are coagulated by bipolar diathermy during the dissection. The next sub-step involves the dissection of the uterine artery and the anterior portion of the internal iliac arter. The branches of the internal iliac artery dissected and are sealed, before transection, with diathermy, vessel sealer, or sutures.

Then the most difficult and time-consuming dissection takes place and this is that of the ureter, between the outlet of the tunnel and to the inlet into the bladder. In this area, there are typically venous branches and possibly also parts of the uterine veins, which will form the main outflow from the organ. Thus, it is essential to preserve all uterine veins, which may also angle up towards the bladder. A rubber sling, secured with Hemoclips®, around the ureter will enable traction and positioning of the free ureter, to facilitate dissection. Great care has to be taken to avoid damage to any uterine vein or important branch. The next surgical step is focused on the dissection of the internal iliac vein in the region of the inlet(s) of the uterine vein(s). During this procedure, Hemoclips®, secured by sutures, or/and vessel sealer as well as bipolar diathermy, will be used on branches before transection. The technique for preventing blood leakage from a branch before severance depends on size of the vein, where clips with securing suture should be used for larger branches. A unilateral dissection of the pelvic sidewall with a totally freed ureter plus appropriate vascular pedicles is then completed, and all the steps will be repeated on the contralateral side, which by our preference is the left side.

After completion of the bilateral pelvic sidewall dissections, a bilateral salpingectomy is performed, and the bilateral dissection of the uterine branch of the utero-ovarian vein is carried out. This upper venous segment will be part of the graft and may be used as an extra venous outflow at UTx. The surgery is then directed to the posterior and caudal aspect of the uterus by initially opening the peritoneum of the pouch of Douglas. Dissection is then performed caudally to separate the rectum from the posterior aspect of the vagina. The uterosacral ligaments are at that time divided. The uterus is at this stage attached only to the vagina and the six vascular pedicles.

The last step in the procurement starts by opening the vagina, using bipolar diathermy and scissors, with the aim to acquire a 2-cm vaginal cuff on the uterine side. The large vessels are then clamped accordingly with a laparoscopic two-row staple instrument in the order of arteries followed by veins. Then a laparoscopic specimen bag is introduced through a laparoscopic port and the uterus is placed inside the bag. The bag is extracted through the vagina. The vaginal cuff is closed from the abdominal side by a continuous V-lock suture. The stumps of the large vessels are then checked for haemostasis. All instruments and ports are then extracted and the port sites on the skin are sutured by skin sutures.

Uterus transplantation—open surgery technique

A sub-umbilical midline incision is used for this laparotomy surgery. The preparatory surgery, before the uterus is lifted in, differs considerably between women with MRKHs and women having undergone hysterectomy or when hysterectomy is part of the transplantation procedure. The different preparatory techniques are described separately below.

In an MRKHs patient, an elongated uterine rudiment is often present just cranial and posterior to the dome of the bladder top, and uterine-tissue buds are often seen on the pelvic sidewalls. The ovaries with connected oviducts are typically located lateral to the external iliac vessels at a cranial position. Initially, the external iliac vessels are separated and dissected free for a distance of around 5–7 cm. The round ligaments are cleaved lateral to the lateral uterine rudiments, in order to position the rudiments so that they do not interfere with the vascular pedicle of the graft after anastomosis to the external iliac vessels. After the preparations on the pelvic sidewalls, surgery is directed towards the vaginal vault, which is typically covered by the bladder and the midline rudiment uterine tissue. Large variations exist in the anatomy and size of the uterine rudiment in MRKHs patients. In patients with unilateral kidneys, the rudiment is typically positioned towards the side of the existing ureter and the vaginal vault will be angled towards that side and with a hypoplastic utero-sacral ligament on the contralateral side. During the dissection of the vaginal vault we use a sphere-shaped vaginal probe to present the vault and to facilitate the surgery. Pressure of the probe should be towards the umbilicus and thereby the edge between the frontal aspect of the midline uterine rudiment and the bladder is easily identified so that the bladder can be dissected free from the frontal aspect of the vaginal vault. The midline rudiment uterine tissue is cleaved over the vault by monopolar electrocautery to acquire full exposure of the top of the vagina. Then the rectum is separated from the posterior of the vagina. The area over the vault, with dissection down to the fascia, should be around 4 and 5 cm in transverse and longitudinal directions, respectively. This will allow good access when performing vaginal–vaginal anastomosis after vaginal opening, which is a late step in order to minimize

bacterial contamination in the pelvis of the immunosuppressed recipient. Fixation sutures by non-absorbable monofilament (1-0 polypropylene), for later structural support, are attached to the utero-sacral ligaments, the round ligaments, and in the bisected uterine rudiments.

Concerning non-MRKHs patients as uterus recipients, they can either have undergone hysterectomy or have a present, malfunctioning uterus. Typically, such a uterus will be removed at the same surgical session as the transplantation procedure. In these types of patients, the dissection and clearance of the external iliac vessels are similar to MRKHs patients (see above). In the totally hysterectomized recipient, post-surgical adhesions and abnormal positions of ovaries may present some challenges. Dissection of the vaginal vault should be performed by standard procedures, so the vaginal vault is solely covered by fascia tissue. The low and lateral fixation sutures in the hysterectomized patient should be placed through the fibrous tissue lateral to the top of the vagina, to mimic the cardinal ligaments. In a subtotal hysterectomized patient, the cervical stump should be removed immediately before the uterus graft is positioned inside the pelvis since the vagina is opened during cervical removal. In a woman that has a full non-functional uterus, a total hysterectomy is performed as part of the preparatory surgery. In such a case, the vaginal vault will be opened and can then be temporarily closed. It is possible to use parts of the uterine arteries and deep uterine veins of the uterus to be removed, for later coupling to the vessels of the graft.

After all preparatory surgery, the chilled and flushed uterine graft is positioned in its anatomical position, within the pelvis of the recipient. Vascular anastomoses are established end-to-side between the internal iliac segments of the graft and the external iliacs of the recipient. The anastomoses are sutured continuously with a polypropylene suture of size 5-0 to 7-0 depending on vessel size and starting with the vein(s) on one side and thereafter the artery. Clamps may be used proximal in the graft after each anastomosis to check for leakage, and extra sutures may be needed for haemostasis over any anastomosis site. After the four to six anastomoses have been completed, clamps are removed to allow uterine reperfusion and for haemostatic control.

The vagina is then opened with a sagittal incision of around 5 cm, with the vaginal probe pushed upwards. End-to-end vaginal anastomosis is then performed between the vaginal vault of the recipient and the vaginal rim of the graft using a continuous 2-0 resorbable suture. The previously performed preparation of fixation sutures in the recipient is then utilized with fixations accordingly (see above). The peritoneal bladder flap of the graft is sutured on top of the bladder of the recipient, as extra structural support and in order to avoid intestinal herniation in front of and at the sides of the transplanted uterus. After ensuring adequate uterine blood perfusion and haemostasis, the midline incision is closed by standard technique.

Uterus transplantation—robotics technique

We have the first experience of totally robotic UTx surgery in a recipient by surgery performed in the second half of 2021. In this advanced procedure, transplantation surgeon, with experience of robotic kidney transplantation, is the main surgeon during uterine insertion, and gynaecologist is the main surgeon in preparation, vaginal anastomosis, and uterine fixation. Key personnel also include the assisting laparoscopist and a surgeon to manoeuvre the vaginal probe during the preparatory surgery, vaginal opening, and during vaginal anastomosis. A robotic system with a four-arm set-up with dual consoles is recommended,

and additionally there should be two laparoscopic ports to enable ideal insertion of sutures and to assist with suction and clamps. A robotic optic scope of 0°, with the possibility to shift to 30°, is recommended, and the port for the camera may be altered during the procedure, in order to acquire optimal vision at the side of anastomosis surgery. The recipient should be positioned in a Trendelenburg position (angle 20°–25°), and side-docking (45°) towards the patient's hip is recommended to enable optimal assistance and manipulation of the vaginal probe. Maryland forceps, monopolar curved scissors, a variety of needle drivers for the various anastomosis, and prograsp forceps are the instruments which are used in this recipient procedure. The camera port and another port is at the level of the umbilicus and the two additional robotic ports are down towards the fossa on each side.

A meticulous back table preparation of the uterus is essential to get four optimal sites on the graft vessels for anastomosis surgery, which is more time consuming by robotics as compared to open surgery at this initial stage. Thus, preparation may include to join two venous outflows on one side to create on distal end of the venous pedicle. Preparatory surgery of the recipient is similar to open surgery but with different surgical instruments used. We describe our own experience in an MRKH patient. After robot docking, the surgery starts with dissection of the external iliac vessels for a distance of around 10 cm, which is a somewhat longer distance than in open surgery. The reason for that is that good access to the vessels is needed since the uterus is not easily repositioned during surgery, in contrast to in an open surgical procedure. The bilateral dissection and separation of external iliac arteries and veins is performed mainly with monopolar curved scissors and Maryland forceps. The same instruments are then used to cleave the midline uterine rudiment, dissect it off the bladder dome, expose the vagina vault, divide the round ligaments, and, if needed, reposition the lateral uterine buds on the pelvic sidewalls. These surgical steps are essentially as in open surgery (see above) but with different instruments used.

In order to position the uterine graft optimally inside the pelvis, there are two possible alternatives to bring the uterus into the abdomen of the recipient. This can be either through a small abdominal incision or through the vagina. In our opinion, it is not advisable to use the vaginal route, even if the uterus is placed inside a laparoscopic bag, because the typical narrow vagina of an MRKHs patient would most likely compress the uterine graft, and especially the delicate vascular pedicles, during insertion into the abdomen. Moreover, the four vascular pedicles should be positioned optimally on the graft at back-table preparation, and it would be difficult to keep such a positioning at vaginal uterine insertion, as already described. We used the abdominal route by a 5- to 6-cm supraumbilical midline incision, which after insertion of the uterus into the pelvis was covered by a Gelport®. The uterus was wrapped inside a gauze and manually (small size hand) brought into its optimal pelvic position easily. After that, a tubing for continuous distribution of chilled preservation solution on the surface of the uterus was inserted through the Gelport®, in order to decrease warm-ischemic injury during the robotic anastomosis surgery. This robotic anastomosis surgery, although restricted to four anastomosis sites, takes more time than that of open anastomosis surgery, and the chilling of the organ will keep the organ cool for a longer time than without this device.

The vascular anastomosis surgery preferable starts on the right side with the end-to-side venous anastomosis with two continuous sutures (Goretex, CV6 needle), and then the same procedure is done for the arterial anastomosis. Needle driver and forceps

are used. Sutures are brought into the abdomen via a laparoscopic port. Clamps are placed proximally towards the graft vessel, ensuring patency and that no leakage exists over the vascular anastomosis site. The procedure is then repeated on the contralateral side and when all anastomosis sites are completed, all vascular clamps are removed, and the uterus is perfused. When haemostasis and perfusion of the uterus is secured, the gynaecologist takes over for vaginal surgery and further fixation. The vagina is opened by monopolar diathermy to create a sagittal incision of around 5 cm in length during pressure on the spherical vaginal probe. The vaginal anastomosis is then performed robotically by a continuous V-lock 2-0 resorbable suture, starting at a lateral aspect and suturing the posterior aspect of the vagina from the inside and the anterior aspect of the vagina from the outside. Uterine fixation is performed by suturing the round ligaments and by oversewing the recipient's bladder with the large bladder peritoneal flap of the graft. After once again making sure that adequate organ perfusion and haemostasis exist, all instruments and ports are extracted. Port incisions are closed by skin sutures, and the Gelport® incision is closed by mass-suture, subcutaneous suture, and skin suture.

Uterus transplantation results

Reports of deceased donor uterus transplantations

Results concerning surgical outcome are available from altogether 11 DD UTx cases (Table 66.1), with the first taking place in 2011 [19]. These publications included five DD UTx conducted in the Czech Republic [20], four cases in the United States [21–23], and single cases in Brazil [24] and Turkey [19]. The overall surgical success, which we define as post-transplant normal blood flow, and regular menstruations, in these 11 DD UTx procedures was 64%. All surgeries in DDs and in recipients of DD uteri have been by laparotomy.

Reports of live donor uterus transplantations

Results concerning surgical outcome are available from altogether 51 LD UTx cases (Table 66.1), with the initial taking place in 2000 [25]. There are 18 published LD UTx cases from one trial in the United States [23], 17 in Sweden [2, 26, 27], five in the Czech Republic [20], and four each in India [28, 29] and Germany [30]. Reports of single LD UTx cases exist from Saudi Arabia [25], China [31], Lebanon [32], and Spain [33].

Donor hysterectomy was first only by open surgery but since then this procedure has been performed both by traditional laparoscopy and robotic-assisted laparoscopy. Recipient LD UTx surgery has been by open surgery in all published cases, but by robotics in our latest UTx case (unpublished observation). Surgical success of LD UTx has been 78% (Table 66.1), with a slightly higher success rate in minimal invasive LD UTx (89%) than in laparotomy LD UTx (73%).

Complications in recipients

Concerning recipients, there are few direct surgery-related complications reported. However, the rate of graft loss by transplantectomy because of low blood flow has been fairly high (see above) but is likely to decrease in the future by learning and stricter inclusion criteria. The surgical time of recipient surgery has been around 4–5 h. Some cases of vaginal stenosis over the end-to-end anastomosis between the vaginal rim of the graft and the vaginal vault of the recipient have been reported. The incidence of vaginal stenosis in the Swedish studies was 14% among successful grafts

TABLE 66.1 Summary of Published (n = 62) Uterus Transplantation (UTx): Data on Surgical Success (SS), Rate of Major Post-Operative Live Donor Complications (DC), and Rate of Surgery-Related Post-Operative Complications in Recipients with Successful Grafts (RC)

Type of UTx	Country	UTx Year(s)	n	RC	SS	DC
DD	Turkey	2011	1	0/1	1/1	-
DD	Czech Rep.	2016–2018	5	2/3	3/5	-
DD	USA	2016–2017	2	0/1	1/2	-
DD	Brazil	2016	1	0/1	1/1	-
DD	USA	2017	2	0/1	1/2	-
LD laparotomy	USA	2016–2019	13	1/8	8/13	2/13
LD laparotomy	Germany	2016–2019	4	0/4	4/4	0/4
LD laparotomy	Lebanon	2018	1	0/1	1/1	0/1
LD robotics	China	2015	1	0/1	1/1	0/1
LD robotics	Sweden	2017–2019	8	2/6	6/8	1/8
LD robotics	USA	2019	5	0/5	5/5	2/5
LD laparoscopy	India	2018–2019	4	0/4	4/4	0/4
Summary		2000–2019	62	9/47	47/62	9/51

Abbreviations: LD, live donor; DD, deceased donor.

in the laparotomy study [2] and 33% in the Swedish robotic UTx study [26, 27]. In the Czech mixed LD–DD UTx trial [20], vaginal stenosis occurred in 57%. Most likely there exist other cases of vaginal stenosis, which are not reported. Vaginal stenosis makes it difficult to obtain cervical biopsies for rejection diagnosis and in embryo transfer (ET). Correction of vaginal stenosis have been by diathermic incisional surgery, forced dilation, and stent, or a combination of these methods.

Graft rejection is frequently seen after UTx, with the majority being minor or moderate in severity [34] and reversible by increased immunosuppression. Occasional cases of severe rejection have been reported [22, 34]. There is no reported case of uterine rejection leading to removal of the graft.

Complications in live donors

The surgery of the LD is complex and with a long duration, with common surgical times from 8 to 12 h [2, 20, 23, 26, 30]. In the Swedish laparotomy study [2], one donor developed a unilateral uretero-vaginal fistula, which was repaired four months later. No other serious post-operative complication was reported among the nine laparoscopic LDs of that trial [20]. In the Czech trial [20], two out of five laparoscopic LDs had major complications. One patient developed urinary bladder hypotonia and this was treated by a suprapubic catheter and the other patient had a ureteric laceration, which was repaired at primary surgery and treated with a ureteric JJ stent. In the LD trial in the United States [23], two major post-surgical complications occurred among the 13 laparoscopic LDs. One patient was treated under anaesthesia for faecal impaction and another underwent surgery for vaginal-vault prolapse.

In robotic LD surgery, one of the eight donors in the Swedish robotic trial had a major post-operative complication, which was pyelonephritis with hydronephrosis, and this was treated with antibiotics and a temporary ureteric JJ stent [26]. In the LD UTx trial in the United States, two out of five operated patients acquired serious post-operative complications [23]. One woman developed a ureteric blood clot with secondary hydronephrosis and another

woman acquired bilateral ureteric-vaginal fistulae. Both were treated by ureteral JJ stents [23].

Efficacy of IVF and obstetrical outcomes in uterus transplantation

The outcome endpoints which should be evaluated in the setting of UTx, which is combined with IVF, are pregnancy rate per ET, live birth rate per ET, since the uterine graft will only be carried for a restricted time until hysterectomy is performed, the cumulative clinical pregnancy rate and cumulative live birth rate per attempted UTx procedure, and per surgically successful UTx procedure with ETs, should also be given. Important obstetrical endpoints are pregnancy duration, birthweight, rates of pregnancy complication, preterm birth, neonatal complications, as well as occurrence of congenital malformation.

IVF treatment before uterus transplantation

In vitro fertilization (IVF) is used as a routine in UTx patients, and has been performed before UTx in all published UTx cases, except in the original case from year 2000 [25]. In the latter case, the oviducts were included in the graft but later cases have not included oviducts. Reasons for exclusion of oviducts are to avoid risk of blocked tubes or tubal ectopic pregnancy, with the risks likely to be considerably increased after UTx.

Initiating IVF treatment prior to the UTx procedure has undeniable benefits. This will confirm a fertility potential in the recipient. It will also minimize the risks of iatrogenic injuries related to oocyte retrieval after UTx surgery and reduce the exposure time of immunosuppressive therapy. In AUFI patients with no uterus and hence no menstrual bleeding, a physiological function of the hypothalamic–pituitary–gonadal axis should be confirmed before IVF. Assessments of luteinizing hormone (LH), follicle-stimulating hormone (FSH), oestradiol, and progesterone over one or two months should be performed. Estimating the

ovarian reserve by measuring serum anti-Müllerian hormone (AMH) level is valuable before starting the stimulation protocol, especially when no optimal ultrasound images of ovaries can be obtained, as in several patients with MRKH, where the ovaries may have a high and lateral position. Also concerning MRKH patients, which have been the vast majority of UTx patients so far, studies indicate that women with type B (with urinary tract malformations such as single kidney) have lower AMH, lower antral follicle count, and decreased ovarian response to gonadotropins, as compared to MRKH women of type A [35].

The ovarian stimulation protocol used for the first UTx trial in Sweden [36] was a long gonadotropin-releasing hormone (GnRH) agonist protocol with downregulation from mid-to-late luteal phase, as estimated from hormone measurements. This was followed by daily injections of FSH and/or human menopausal gonadotropin (hMG) and finally human chorionic gonadotropin (hCG) was given to trigger oocyte maturation. In that study, transabdominal oocyte retrieval was performed in four women and transvaginal retrieval in five women. Eight women needed two IVF cycles to accumulate the stipulated 10 high-quality embryos to be cryopreserved before IVF. Details of the accumulated embryos in each cycle, along with background data of the women of the study, have recently been published [36].

In our second study, the Swedish robotic UTx trial [27], a random-start stimulation protocol [37] was used. By this method, daily injections of hMG/FSH were started simultaneously with GnRH-antagonist injections (no matter the day of menstrual cycle) and oocyte maturation was always triggered by GnRH-agonist. Oocytes were retrieved 36 h after injection of GnRH-agonist trigger. Retrieval was vaginally in seven women and transabdominally in one woman. One or two IVF cycles were needed to accumulate the stipulated eight high-quality blastocysts for pre-UTx cryopreservation [36]. The random-start stimulation protocol showed many advantages compared to the former protocol for the MRKH patients. Stimulation could start anytime in the menstrual cycle, there was almost no risk of ovarian hyperstimulation syndrome (OHSS), and it optimized the number of retrieved and cryopreserved oocyte/embryos per started stimulation cycle.

In both Swedish studies [27, 36] methodology for preparation and incubation of sperms were standard IVF, and ICSI was only used when the sperm sample of the day of oocyte recovery was below the normal ranges for either motility, concentration, or total counts. The majority of embryos were cryopreserved at the blastocyst stage.

IVF treatment after uterus transplantation

There is also experience of post-UTx. Thus, some UTx patients, with good ovarian reserve as well as stable and functioning transplanted uteri, required post-UTx IVF treatment after utilizing all their spare embryos without accomplishing live birth or for other specific reasons, as stated below.

Specific challenges with post-UTx IVF can be the altered position of the ovaries after surgery and a changed pelvic vasculature, which may present problems at oocyte retrieval. Thus, intra-abdominal bleeding may occur more easily and can be more severe in post-UTx as compared to pre-UTx IVF. Furthermore, the susceptibility to pelvic infections in conjunction with oocyte retrieval, due to the immunosuppressed state of the woman, is greater. The major advantage of post-UTx IVF, in comparison to pre-UTx IVF, is that the woman has menstruations and that a

stimulation protocol, whether antagonist or agonist protocol, can be started at the optimal time.

There are two scientific reports of post-UTx IVF stimulation with oocyte retrieval. In the German UTx trial, the reason for post-UTx IVF was exhausted pools of embryos that had been cryopreserved before UTx, due to multiple implantation failures of pre-UTx embryos in one case [30]. Another patient of the same study had mature oocytes, but not embryos, cryopreserved before UTx. The oocytes, which had been cryopreserved by slow-freezing, did not survive thawing. A GnRH-antagonist protocol was used in both these post-UTx cases with vaginal oocyte retrieval, even though the ovaries had been relocated lateral to the external iliac vessels at ovary pexy during transplantation [30]. No complication was seen.

In the original Swedish study with transplants in 2013 [36], post-UTx IVF was performed in three out of seven women with successful grafts. The reasons for IVF treatments after UTx were in two cases exhausted pools of pre-UTx embryos, and in the third case IVF was done after UTx because of separation from her male partner within the first year after UTx and hence a need for IVF with donor sperms. The latter case suggests that a mix of unfertilized oocytes and embryos should be cryopreserved before UTx, in case the marital status of a recipient changes for any reasons, and the embryos cannot be used anymore.

Embryo transfer in uterus transplantation patients

The time for the first ET post-UTx was 12 months after UTx in the Swedish laparotomy UTx study [36], in order have stable immunosuppression and low risk for graft rejection. Since then, the interval has been shortened by several groups to an interval between 3 and 10 months post-UTx, with the time period decided by the rejection pattern and clinical pattern of each patient.

Single embryo transfer (SET) should be compulsory in a UTx setting, since transfer of multiple embryos will markedly increase the risk of a pregnancy with twins or triplets, with the coupled risk for obstetric, neonatal, and postnatal complications. Moreover, the pelvic fixation and vascular supply of a transplanted uterus may not withstand a multiple pregnancy, with a substantially larger uterus. To our knowledge, strict SET policies have been present in all trials except in two trials with single patients receiving two or three embryos at one occasion [20, 31]. However, these ETs with multiple embryos did not result in pregnancies with multiple fetuses.

The vast majority of the SET procedures of the Swedish patients of the laparotomy and robotic UTx studies were performed in natural cycles [37, 38]. The LH surge was detected by self-examinations of urinary LH and day 2 embryos or blastocysts were transferred in the early afternoon of day LH +3 and LH +6, respectively. In a few patients with non-detectable LH signals or with irregular cycles, programmed cycles were used.

There exist some special concerns of ET after UTx, as compared to the normal situation. The length of the cervix of a transplanted uterus tends to gradually increase after transplantation, and we have encountered 10-cm-long cervical canals some years after UTx. This post-UTx cervical hypertrophy may be secondary to increased uterine blood flow after UTx, caused by a shunting of all blood through the well-sized anterior portion of the internal iliac to the uterus, since other branches are ligated. Furthermore, a transplanted uterus is often attached to the abdominal wall by adhesions and may therefore be positioned into an extreme anteflexion

position. This will give a sharp angle between the uterine cavity and the cervical canal and, moreover, stenosis over the vaginal–vaginal anastomosis may present problems, as discussed above. The cervical length, possible influence of vaginal stenosis, and degree of cervico-uterine angulation should be examined by a mock transfer before any proper ET, and examinations can also include transvaginal ultrasound examination and/or office hysteroscopy. An extra-long ET catheter may be needed, and also with the aid of a guidewire. Obviously, ET should be performed under transvesical-abdominal ultrasonic guidance, to ensure optimal embryo position.

Reproductive outcome after UTx

At present, only one complete report has been published on true reproductive and obstetric outcome after UTx in combination with IVF, comprising final results of one trial [36]. This data is from the laparotomy UTx trial of Sweden, with surgeries performed in 2013 and with all patients having undergone hysterectomy in early 2020 [36, 39]. Seven out of nine women underwent surgically successful transplants and they started ET attempts 12 months post UTx.

Six out of seven women gave birth, with three of the women having two children at separate times. Thus, in total, nine babies were born in this cohort [36]. The total number of ETs was 46 with a clinical pregnancy rate per ET of 32.6%. The live birth rate per ET was 19.6% in the seven women undergoing ET and 30.0% in the six women who gave birth. The low live birth rate was partly due to the fact that one patient had in total 16 ETs, but resulting in six spontaneous abortions and unfortunately no live birth. The six spontaneous abortions were in gestational weeks 7–8, (n = 4) and in gestational week 15 (n = 2). Histopathology of the two late miscarriages showed acute chorioamnionitis. The cumulative live birth rate for the seven women with viable grafts was 86% and for the nine attempted UTx procedures, the cumulative live birth rate was 67%.

Data from three not yet completed trials give some further indications regarding the clinical pregnancy rate and live birth rate. In the Czech UTx trial [20], seven transplants (three DD and four LD) were successful, with 50 ETs reported and a clinical pregnancy rate per ET of 14%. In the German LD UTx trial [30], seven ETs were performed in two patients with a clinical pregnancy rate per ET of 43%. In the large mixed LD–DD UTx trial in the United States, the clinical pregnancy rate so far is 63% [40].

Obstetrical and neonatal outcome after UTx

There are data published on pregnancy and neonatal outcomes of 31 live births after UTx (Table 66.2). The 31 live births are from trials in Sweden [36, 38], the United States [41], Brazil [24], the Czech Republic [20], Germany [30], China [42], and Lebanon [32]. Outcome during the first years after birth is only available from the Swedish study, where a cohort of nine babies were followed in detail during the initial two years of life [36]. All births have been by caesarean section.

Nine births were reported in the Swedish trial [36] as well as in the laparotomy UTx part of the Baylor trial [41]. The births of the US trial (41) took place between gestational weeks 30+6 and 38+0, with the 30+6 birth being due to preterm labour (PTL). The nine births of the Swedish trial [36] occurred between 31+6 and 38+0, with the 31+6 birth being due to pre-eclampsia (PE). In that trial [36], five live births resulted from IVF treatments performed after UTx and from IVF and cryopreservation prior to UTx.

In the Swedish trial [36], we initially aimed for delivery by caesarean section around 35 full gestational weeks, but during the course of the study the scheduled delivery was changed to more than 37 full gestational weeks, in order to achieve optimal fetal lung maturation. According to our local routines, IV corticosteroid was administered to accomplish fetal lung maturation if delivery was to occur before week 34+0. The births of all other trials occurred after 34+0 weeks, except two births after robotic donor hysterectomy, as part of LD UTx procedures within the US trial [41], with deliveries at 32+6 [41] and 33+4 [42]. The cause of delivery was PTL in both cases. Eighteen of the 31 deliveries were according to protocol, with delivery times between 34+6 and 38+8, and with 8/18 (44%) being term ($\geq 37+0$). Eleven out of the 31 (35%) live births were associated with respiratory distress syndrome (RDS) in the neonate (Table 66.2). Ten out of 11 (91%) of the RDS cases were in children born prematurely ($< 37+0$) and three of the ten premature RDS cases were in children delivered per protocol. Thus, there is certainly a need of an extension of per protocol deliveries until 37 full weeks and more. The preterm per protocol timing of the delivery in some trials most likely represents a compromise between achieving adequate maturation of the child and avoiding possible UTx-related obstetric complications during late pregnancy.

There was a higher rate of pre-eclampsia (PE) than in a normal IVF population, with PE in 4/31 (13%). The cause of that is unclear but can be due to the fact that several women had single kidneys and with additional effects by immunosuppression [36, 43]. Furthermore, the rates of placenta previa (PP) and gestational diabetes (GD) were 3/31 (10%) each, which is higher than in a normal population. It remains to be elucidated whether these increased rates of pregnancy complications are still seen in larger UTx materials and in that case what underlying mechanisms exist and if the conditions could be prevented.

In the first UTx trial with nine live births in Sweden, the weight deviations (median [range]) in relation to gestational duration were -1% (-13% to $+23\%$) and Apgar scores at one and five minutes were 9 [3–9] and 10 [8–10], respectively [36]. The cohort of children was followed regarding growth trajectory and health up to two years. Growth was normal considering both weight and length [36]. All children were in good health during the first two years.

Conclusion

This chapter gives an update on the surgical techniques and results of IVF within the rapidly developing UTx field. Uterus transplantation is now in a transition from an experimental procedure into a clinical infertility treatment. However, with fewer than 100 cases performed worldwide and limited long-term follow-up studies, it is important that results of ongoing trials are published and that all cases are prospectively entered into the internal uterus transplantation registry of the International Society of Uterus Transplantation (ISUTx) in order to further develop this treatment into a safe, efficient, and cost-effective infertility treatment for the hundreds of thousands of women in the world with AUFI.

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TABLE 66.2 Reported Pregnancies with Live Births (n = 31) after Uterus Transplantation

Trial	Week of Live Birth	Pregnancy Complication	Indication for Delivery	Apgar (1/5 min)	Neonatal Complication
LD laparotomy (Sweden)	31+6	PE	PE	9/10	RDS
	34+4	ICP	ICP	9/10	RDS
	35+0	-	per protocol	8/8	RDS
	37+0	-	per protocol	9/10	-
	34+4	PE, ICP, PPROM	PE	3/7	RDS
	35+3	PE	PE	9/10	-
	35+6	-	per protocol	9/9	-
	37+1	-	per protocol	9/10	-
	38+0	-	per protocol	9/10	-
	33+1	SCH	low renal function	8/9	RDS
LD laparotomy (USA)	36+6	-	per protocol	9/9	-
	38+0	-	per protocol	9/9	-
	35+6	GD	per protocol	8/8	RDS
	30+6	PTL	PTL	7/8	RDS
	37+2	-	per protocol	8/8	-
	37+0	PP	per protocol	8/9	-
	36+6	PE	PE	8/9	-
LD laparotomy LD (Germany)	35+1	PPROM	PPROM	9/10	RDS
	36+3	GH	GH	8/8	-
LD laparotomy (Czech Rep)	35+3	GD	per protocol	9/10	-
	36+2	PH	per protocol	10/10	-
LD laparotomy (Lebanon)	35+2	PTL	PTL	9/10	-
LD robotics (China)	33+6	SCH	PTL	10/10	-
LD robotics (Sweden)	36+1	-	per protocol	9/10	RDS
LD robotics (USA)	37+0	GH, PH	per protocol	4/8	RDS
	32+4	PP, PTL	PTL	7/8	RDS
	35+6	PTL	PTL	8/8	-
	35+3	PN	per protocol	9/10	-
DD (Brazil)	34+2	PP, PA	per protocol	8/9	-
	37+6	GH	per protocol	9/9	-
	34+6	GD	per protocol	7/9	-

Abbreviations: LD, live donor; DD, deceased donor; GD, gestational diabetes; GH, gestational hypertension; ICP, intrahepatic cholestasis of pregnancy; PA, placenta accreta; PH, polyhydramnion; PTL, preterm labour; PN, pyelonephritis; PP, placenta previa; SCH, subchorionic hematoma; RDS, respiratory distress syndrome.

Conflict of interest

The authors have no conflict of interest.

References

- Bränström M, Diaz-Garcia C, Hanafy A, et al. Uterus transplantation: Animal research and human possibilities. *Fertil Steril*. 2012;97:1269–76.
- Bränström M, Johannesson L, Dahm-Kähler P, et al. First clinical uterus transplantation trial: A six-month report. *Fertil Steril*. 2014;101:1228–36.
- Bränström M, Johannesson L, Bokström H, et al. Livebirth after uterus transplantation. *Lancet*. 2015;14:607–16.
- Sieunarine K, Zakaria F, Boyle D, et al. Possibilities for fertility restoration: A new surgical technique. *Int Surg*. 2005;90:249–56.
- Brett KM, Higgins JA. Hysterectomy prevalence by Hispanic ethnicity: Evidence from a national survey. *Am J Publ Health*. 2003;93:307–12.
- Quinn MA, Benedet JL, Odicino F, et al. Carcinoma of the cervix uteri. FIGO 26th annual report on the results of treatment in gynecological cancer. *Int J Gynaecol Obstet*. 2006;95(Suppl 1):S43–103.
- Creasman WT, Odicino F, Maisonneuve P, et al. Carcinoma of the corpus uteri. FIGO 26th annual report on the results of treatment in gynecological cancer. *Int J Gynaecol Obstet*. 2006;95(Suppl 1):S105–43.
- Kwee A, Bots ML, Visser GH, et al. Emergency peripartum hysterectomy: A prospective study in the Netherlands. *Eur J Obstet Gynecol Reprod Biol*. 2006;124:187–92.
- Glaze S, Ekwalanga P, Roberts G, et al. Peripartum hysterectomy: 1999 to 2006. *Obstet Gynecol*. 2008;111:732–8.
- Marshall LM, Spiegelman D, Barbieri RL, et al. Variation in the incidence of uterine leiomyoma among premenopausal women by age and race. *Obstet Gynecol*. 1997;90:967–73.
- Herlin MK, Petersen MB, Bränström M. Mayer-rokitansky-küster-hauser (MRKH) syndrome: A comprehensive update. *Orphanet J Rare Dis*. 2020;15:214.
- Galliano D, Bellver J, Diaz-Garcia C, et al. ART and uterine pathology: How relevant is the maternal side for implantation? *Hum Reprod Update*. 2015;21:13–38.
- Al-Inany H. Intrauterine adhesions. An update. *Acta Obstet Gynecol Scand*. 2001;80:986–93.
- Fernandez H, Al-Najjar F, Chauveaud-Lambling A, et al. Fertility after treatment of Asherman's syndrome stage 3 and 4. *J Minim Invasive Gynecol*. 2006;13:398–402.

15. Critchley HO, Wallace WH. Impact of cancer treatment on uterine function. *J Natl Cancer Inst Monogr.* 2005;64–8.
16. Vernaeve V, Bodri D, Colodron M, et al. Endometrial receptivity after oocyte donation in recipients with a history of chemotherapy and/or radiotherapy. *Hum Reprod.* 2007;22:2863–7.
17. Saravelos SH, Cocksedge KA, Li TC. Prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure: A critical appraisal. *Hum Reprod Update.* 2008;14:415–29.
18. Grimbizis GF, Camus M, Tarlatzis BC, et al. Clinical implications of uterine malformations and hysteroscopic treatment results. *Hum Reprod Update.* 2001;7:161–74.
19. Ozkan O, Akar ME, Ozkan O, et al. Preliminary results of the first human uterus transplantation from a multiorgan donor. *Fertil Steril.* 2013;99:470–6.
20. Fronek J, Kristek J, Chlupac J, et al. Human uterus transplantation from living and deceased donors: The interim results of The first 10 cases of The Czech trial. *J Clin Med.* 2021;10:586.
21. Flyckt RL, Farrell RM, Perni UC, et al. Deceased donor uterine transplantation: Innovation and adaption. *Obstet Gynecol.* 2016;128:837–42.
22. Flyckt R, Falcone T, Quintini C, et al. First birth from a deceased donor uterus in the United States: From severe graft rejection to successful cesarean delivery. *Am J Obstet Gynecol.* 2020;223:143–51.
23. Testa G, McKenna G, Bayer J, et al. The evolution of transplantation from saving lives to fertility treatment: DUETS (Dallas UtErus transplant study). *Ann Surg.* 2020;272:411–17.
24. Ejzenberg D, Andraus W, Mendes LRBC, et al. Livebirth after uterus transplantation from a deceased donor in a recipient with uterine infertility. *Lancet.* 2019;392:2697–704.
25. Fageeh W, Raffa H, Jabbad H, et al. Transplantation of the human uterus. *Int J Gynecol Obstet.* 2002;76:245–51.
26. Brännström M, Kvarnström N, Groth K, et al. Evolution of surgical steps in robotics-assisted donor surgery for uterus transplantation: Results of the eight cases in the Swedish trial. *Fertil Steril.* 2020;114:1097–107.
27. Brännström M, Dahm-Kähler P, Ekberg J, et al. Outcome of recipient surgery and 6-month follow-up of the Swedish live donor robotic uterus transplantation trial. *J Clin Med.* 2020;9:2338.
28. Puntambekar S, Telang M, Kulkarni P, et al. Laparoscopic-assisted uterus retrieval from live organ donors of uterine transplant: Our experience of two patients. *J Minim Invasive Gynecol.* 2018;25:622–31.
29. Puntambekar S, Puntambekar S, Telang M, et al. Novel anastomotic technique for uterine transplant using utero-ovarian veins for venous drainage and internal iliac arteries for perfusion in two laparoscopically harvested uteri. *J Minim Invasive Gynecol.* 2019;4:628–35.
30. Brucker SY, Strowitzki T, Taran FA, et al. Living-donor uterus transplantation: pre- intra- and post-operative parameters relevant to surgical success, pregnancy and obstetrics with live births. *J Clin Med.* 2020;9:2485.
31. Wei L, Xue T, Tao KS, et al. Modified human uterus transplantation using ovarian veins for venous drainage: The first report of surgically successful robotic-assisted uterus procurement and follow-up for 12 months. *Fertil Steril.* 2017;108:346–56.
32. Akouri R, Maalouf G, Abboud J, et al. First live birth after uterus transplantation in the middle East. *MEFS J.* 2020;25:30–7.
33. Carmona F, Rius M, Díaz-Feijoo B, et al. Uterine transplantation. First viable case in southern europe. *Med Clin (Barc.).* 2021;26:156:297–300.
34. Mölne J, Broecker V, Ekberg J, et al. Monitoring of human uterus transplantation with cervical biopsies: A provisional scoring system for rejection. *Am J Transplant.* 2017;17:1628–36.
35. Raziel A, Friedler S, Gidoni Y, et al. Surrogate in vitro fertilization outcome in typical and atypical forms of Mayer-Rokitansky küster-hauser syndrome. *Hum Reprod.* 2012;27:126–30.
36. Brännström M, Dahm-Kähler P, Kvarnström N, et al. Reproductive, obstetrical and long-term health outcome after uterus transplantation: Results of the first clinical trial. *Fertil Steril.* 2022; in press
37. Cakmak H, Katz A, Cedars MI, et al. Effective method for emergency fertility preservation: Random-start controlled ovarian stimulation. *Fertil Steril.* 2013;100:1673–80.
38. Brännström M, Dahm-Kähler P, Kvarnström N, et al. Live birth after robotic-assisted live donor uterus transplantation. *Acta Obstet Gynecol Scand.* 2020;99:1222–9.
39. Karlsson CC, Dahm-Kähler P, Kvarnström N. Hysterectomy after uterus transplantation and detailed analyses of graft failures. *Acta Obstet Gynecol Scand.* 2021; epub Dec 21.
40. Putman JM, Zhang L, Gregg AR, et al. Clinical pregnancy rates and experience with in vitro fertilization after uterus transplantation: Dallas Uterus transplant study. *Am J Obstet Gynecol.* 2021;225:155.e1–155.e11.
41. Johannesson L, Testa G, Putman JM, et al. Twelve live births after uterus transplantation in the Dallas uterus transplant study. *Obstet Gynecol.* 2021;137:241–9.
42. Huang Y, Ding X, Zhang G, et al. Report of the first live birth after uterus transplantation in China. *Fertil Steril.* 2020;114:1108–15.
43. Brännström M, Diaz-Garcia C, Johannesson L, et al. Livebirth after uterus transplantation – Author's reply. *Lancet.* 2015;385:2352–3.

Introduction

In the context of offering assisted reproductive treatments (ART), the viral infections of most concern are human immunodeficiency virus types 1 and 2 (HIV), hepatitis B (HBV) and hepatitis C (HCV). Less frequently encountered, but routinely screened for during donor programmes in those deemed at risk, are human T-lymphotropic virus I and II. Zika (ZIKV) also has an impact on ART programmes, as a result of global travel trends and fertility tourism, and needs to be considered in managing ART programmes in at-risk groups, especially in the context of gamete donation. More recently, as a result of the global pandemic, severe acute respiratory coronavirus syndrome 2 (SARS-CoV-2) has emerged as a virus of concern, both in managing risk during ART programmes and later during pregnancy. Monkeypox (MPXV) has also recently emerged as a virus of concern in certain high-risk groups, in particular men having sex with men, and risk should be considered in sperm gamete donation and third-party reproductive programmes.

In managing viral transmission risk in the ART setting, the following must be considered:

1. Appropriate viral screening prior to ART, defined by patient demographics and risk factors according to published national guidelines to ensure compliance with regulatory standards. A full review of such regulation, country by country, is beyond the remit of this chapter. ART centres are expected to have a quality management system (QMS) in place with screening requirements as stipulated by national standards defined within their standard operating procedures to deal with the consequences of a positive screening result.
2. Minimizing horizontal viral transmission risk during the ART programme in those with known viral infections, to safeguard the patients and their partners, and other patients and staff at the centre and minimizing vertical transmission to the unborn child. This applies to both clinical procedures and laboratory processes.
3. The impact of any given viral infection or viral infection combination on fertility and ART choice and outcome.

This chapter sets out the latest evidence base and available guidance for planning and managing a cost-effective, safe, and ethically sensitive fertility service for patients diagnosed with bodily fluid and respiratory viral infections. For each viral infection, an overview is provided on the virus before setting out treatment requirements and on the potential effect of the virus on fertility and treatment outcome. General principles covering viral screening and the handling of samples from viral positive patients to minimize risk in clinical and laboratory ART practice are covered at the end of the chapter. Whilst it is recognized that local legislation and guidance will ultimately dictate a centre's practice,

the latest published guidance from the Ethics Committee of the American Society of Human Reproduction (ASRM) [1] and the European Society of Human Reproduction (ESHRE) [2] are cited where relevant, and areas of practice where guidance differs is discussed.

Human immunodeficiency virus (HIV)

Overview

HIV, a retrovirus, uses reverse transcriptase to transcribe RNA into DNA. The virus binds to cell surfaces, typically helper CD4⁺ T-lymphotropic, and leads to their progressive depletion over time [3]. This leads to those infected with HIV developing a weakened immune system with reduced ability to fight off infections and some forms of cancer. Once the CD4⁺ count falls below 200 copies, HIV RNA/mL development of acquired immunodeficiency syndrome (AIDS) can occur. Disease progression from initial seroconversion to development of symptoms can be slow, with lapses of up to 15 years, allowing for timely intervention. At the point the lymph nodes become infected with HIV, the virus enters the blood stream and passes into other body fluids, including semen and breast milk [4]. Unprotected intercourse is well established as the predominant form of HIV transmission, with further significant numbers of children becoming infected through vertical perinatal transmission. HIV is measured in blood and other body fluids using polymerase chain reaction assays (PCR) [5].

There are two major types of HIV, referred to as HIV-1 and HIV-2. HIV-1 was the first described strain of HIV and is the more prevalent and virulent. There are more than 100 subtypes or recombinant forms of HIV-1—characterized by their genetic variability—which can affect screening, diagnosis, and response to antiretroviral treatment. HIV-2 is far less prevalent, mainly being found in West Africa, and its transmission is four times less than with HIV-1 due to lower plasma and semen viral loads.

Following infection, HIV RNA first appears in the blood followed by p24 antigen, then HIV IgM antibody, and finally HIV IgG antibody. Routine HIV screening in the clinical setting typically employs the fourth generation HIV test, a serum immunoassay that detects both antibodies against HIV-1 and -2 as well as the p24 antigen which, as an early marker for HIV infection, enables early detection of the virus [6, 7]. The nucleic acid test (NAT) is another useful tool in the ART setting as it can detect HIV-RNA as early as 5–10 days after the primary infection. The main use of this test in ART programmes is in gamete donation screening to exclude seroconversion of an HIV negative individual during treatment or to enable early quarantine release of samples that have been cryopreserved.

Highly active antiretroviral therapy was first introduced in 1995 and observed to halt the natural progression of HIV to AIDS [8]. The antiretroviral combination regimens comprise reverse transcriptase and protease inhibitors, which interrupt viral

replication and slow down and halt CD4⁺ depletion. In the majority of cases, viral replication in infected individuals is completely stopped, leading to undetectable levels of HIV-RNA in blood plasma. As a consequence, CD4⁺ counts recover to normal levels. For individuals diagnosed soon after initial HIV infection and offered early antiretroviral treatment, life expectancy approaches that of uninfected individuals [9, 10], which has led to HIV being defined as a chronic disease [11].

Globally, by the end of 2021, 38.4 million people were living with HIV, equating to 0.7% of the adult population aged between 15 and 49. Two-thirds were living in the World Health Organization (WHO) African region (WHO, 2022). There is still no vaccine available, so effective early implementation of antiretroviral treatment remains key in the global health strategy to reduce advanced disease and transmission. Since 2016, the WHO has recommended that all people living with HIV should have access to lifelong antiretroviral medicine, regardless of clinical status or CD4 count, and that this should be started from the time of diagnosis. By the end of 2021, 28.7 million people living with HIV had access to antiretroviral medicine, equating to 75% of the total world adult population, but only 50% of the child and adolescent population (WHO, 2022).

Patients presenting for ART are now either newly diagnosed with HIV, as a result of pre-treatment screening, or already living with HIV. In the former case, correct management should be immediate referral to an HIV physician for full screening, assessment, and commencement of antiretroviral treatment prior to starting ART. In the latter case, the overwhelming majority will already be on antiretrovirals and have sustained, undetectable HIV viral loads. Very few patients therefore will present for ART with detectable HIV RNA in the serum or semen.

HIV sexual transmission risk

It has long been established that patients living with untreated HIV wishing to reproduce naturally present a high risk of horizontal and vertical transmission. By contrast, when a patient is on antiretroviral treatment, the risk of sexual and horizontal transmission is now considered to be negligible. The concept of zero sexual transmission risk for those on antiretroviral treatment was first published by the Swiss Commission on AIDS in 2008 as a commentary informing Swiss patients and physicians that, under optimal conditions, the risk of HIV transmission appeared to be negligible [12]. Although widely disputed by many experts at the time, a substantial body of evidence published since has endorsed this statement [13]. The HIV Prevention Trials Network 052 study (HPTN 052) was a clinical randomized controlled trial designed to investigate the impact of early antiretroviral-start (instead of delayed) prevented the risk of HIV transmission in HIV serodiscordant or serodifferent couples [14]. The study enrolled 1763 couples in 13 centres across nine countries in Asia, Africa, and the Americas and found no case of transmission from any HIV-infected individual where blood viral load was fully suppressed through antiretroviral treatment. The HPTN 052 study only reported new transmissions in cases where the infected partner was not fully suppressed, occurring at the initiation of treatment or when the treatment was no longer effective at suppressing viral replication. Overall, this study demonstrated a 96% reduction of HIV in heterosexual couples, and observed that early treatment of HIV also reduced other infections in those living with HIV. The European Partner Study reinforced the findings of HPTN 052. This was initially published as a prospective observational study of HIV-serodiscordant couples who informed

their physicians they were practicing sex without condoms and were followed up with six monthly HIV tests in the uninfected partner [15]. After a total follow up of 1238 couple-years, not a single case of transmission from the infected partner under antiretroviral treatment was documented. The Partner study was extended to cases of HIV-positive men having sex with men where the infected partner, who had fully suppressed viral load through antiretroviral treatment, was in a gay or bisexual relationship [16], and final outcomes were published in 2019. Again, there were no reported cases of transmission when the viral load of the HIV infected partner was undetectable. Le Messurier et al. [17] conducted a systematic review and meta-analysis of HIV-serodiscordant heterosexual partners having unprotected intercourse, where the index case was on antiretroviral treatment and had a fully suppressed viral load. No transmissions occurred over 1327 person-years (pooled incidence 0.00 transmissions/100 person-years, 95% CI 0.00–0.28, two studies).

The aforementioned studies have provided robust scientific evidence to support the statement that HIV infected individuals with a fully suppressed, sustained undetectable HIV RNA viral load (<50 copies/mL) through use of antiretroviral treatment are sexually non-infectious. HPTN 052 and the European Partner Study led to the Prevention Access Campaign's undetectable equals untransmittable (U = U) campaign launched in 2016. This international science-based consensus statement has since been endorsed by more than 1000 partners in 105 countries and has not only reduced the stigma surrounding HIV but reassured infected individuals and their partners who wish to conceive that they can and should do so naturally. The studies also led the WHO to recommend that everyone with HIV should start antiretroviral medicine as soon as they are diagnosed.

Natural conception in HIV discordant couples

HIV physicians now advise all HIV-serodiscordant heterosexual couples who are on antiretroviral treatment with a sustained undetectable viral load that, unless a fertility factor exists, natural conception through timed unprotected intercourse should be their first line approach to family building [18–20]. When the partner living with HIV is not on antiretroviral treatment, e.g. newly diagnosed or through choice, they should be advised to start antiviral therapy before trying to conceive. Natural conception should be delayed for at least six months to ensure sustained, undetectable HIV RNA serum levels have been attained [21]. ART is only indicated in those not willing to start antiretroviral therapy or who fail to get full viral suppression or when a fertility factor or unexplained infertility is diagnosed.

Studies assessing the risk and outcome of natural conception in serodiscordant couples with HIV are very reassuring, and more recent studies indicate no need for additional measures such as restricting intercourse to the window of ovulation and use of pre-exposure prophylaxis (PrEP). In an early prospective study of 53 serodiscordant couples in whom the HIV-positive man had been successfully treated with antiretrovirals for more than six months and had undetectable levels of HIV-RNA in the plasma (<50 copies/mL), pregnancy rates of 26% were reported for the first attempt, rising to 66% after five attempts and 75% after 12 attempts. Median female age was 33 years and 244 events of unprotected intercourse took place over the study period [20]. A later Cochrane database review of natural conception in HIV-serodiscordant couples analysed seven observational studies and one randomized controlled trial. No transmissions were noted in couples where the HIV infected partner was on antiretroviral

treatment with an undetectable viral load [22]. In the early studies, PrEP, in the form of tenofovir at the time of the urine LH surge and 24 hours later, was offered to the HIV-negative female partner as well as other safeguards to reduce the residual anxiety [20, 23]. Subsequent studies have shown that when the man has undetectable through antiretroviral treatment, PrEP appears to confer no additional risk reduction in couples attempting to conceive naturally [24]. PrEP is only appropriate in those couples wishing to conceive naturally if the man is not on antiretroviral treatment [23–25].

Assisted reproduction

The decision to offer IVF and ICSI in couples who are either HIV-serodiscordant or HIV-concordant should be based on diagnosed fertility factors or prolonged unexplained infertility, not on HIV status, and the clinical protocols advised should be exactly the same as those who screen negative for HIV or other viral diseases. In serodiscordant couples where the male is HIV positive and in HIV-concordant couples, published guidance advises additional laboratory steps involving semen processing (sperm washing) with swim-up must be followed as discussed later [1, 2]. Clinical workup prior to offering assisted reproduction should include a full medical and social history along with a sexual health screen and fertility screen in both partners, as would be carried out in viral negative patients. Genital lesions and/or infections should be treated before any fertility treatment is envisaged and treatment planned according to underlying fertility issues. A multidisciplinary approach is advised involving reproductive medicine specialist, HIV physician, counsellor, and, in the case of HIV positive women, obstetrician to ensure the couple are fully informed of their options and risks and involved in the treatment planning.

HIV-serodiscordant men and the role of sperm washing

Prior to the publication of HPTN 052 and the European Partner Study, HIV serodiscordant couples in which the male was HIV positive were advised to practice intercourse with condoms and consider sperm washing to conceive to minimize the risk of viral transmission to their partner and future offspring. This technique was first proposed in 1992 by Enrico Semprini [26], several years before the introduction and development of antiretroviral medication. The technique requires centrifugation of freshly ejaculated semen in a 40%–80% colloidal, silica density gradient to separate progressively motile HIV-free sperm from non-spermatozoa cells (NSC) and seminal plasma that remain in the supernatant. In most studies, the sperm is washed twice before performing a swim-up. The technique rests on the fact that HIV is present in seminal fluid and as cell-associated virus in leucocytes and NSC but is not capable of attaching to, or infecting, spermatozoa [27–32]. Quantitative assessment of HIV-1 before and after sperm washing has confirmed that more than 99% of the virus is removed [33], but further testing of the washed sperm fraction is advised through PCR testing for residual HIV RNA as residual HIV-1 has been shown to be present in a small percentage of cases, even when serum viral load is undetectable.

For the vast majority of HIV-serodiscordant men with undetectable viral loads through antiretroviral therapy, sperm washing combined with intrauterine insemination (IUI) is no longer recommended as a means of preventing HIV horizontal transmission, and the couples are encouraged to conceive through unprotected intercourse. Sperm washing combined with IUI does not reduce transmission risk in these couples. It is unnecessarily

invasive, expensive, and can reduce the likelihood of pregnancy due to its negative effects on semen quantity and quality. Sperm washing combined with IUI should, however, be offered to those couples who, despite reproductive counselling, still perceive the risk of HIV transmission through sexual intercourse to be unacceptable, or in the rare cases where the man is unable to achieve stable undetectable viral loads through the use of antiretrovirals. It also has a place in countries where access to antiretroviral medication is still limited. If there are no fertility factors, IUI should be offered in the first instance and *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) considered in combination with sperm washing after six failed IUI cycles or where fertility factors exist.

A more debatable issue is whether sperm washing should be performed when the male partner has achieved an undetectable viral load through antiretroviral treatment but fertility issues necessitate the use of IVF or ICSI to conceive. If serum viral load is undetectable and as a result natural conception through unprotected intercourse poses no risk of horizontal transmission, the logical argument should be that semen preparation as part of the IVF process should not be any different when the sperm provider is HIV positive with an undetectable serum viral load or HIV negative. However, in the latest published guidance from the Ethics Committee of the American Society of Human Reproduction (ASRM) [1] and the European Society of Human Reproduction (ESHRE) [2], both advise that semen from HIV positive men should still in such cases be processed in a density gradient, washed twice, and a swim-up performed. They also advise post-wash HIV PCR testing before the sample is used in treatment. The evidence behind these recommendations is based on studies that have demonstrated poor correlation between HIV in serum and semen and the fact that HIV has been detected in semen of men with sustained undetectable viral loads. Early studies, carried out at a time when few patients were on antiretroviral therapy, suggested a 3%–6% risk of detectable HIV being present in the washed semen sample [34–36], which is why early sperm-washing protocols always included a swim-up and post-wash PCR test. A later study of 186 seminal samples from men with undetectable viral load as a result of antiretroviral treatment identified that 18 (9.7%) had demonstrable virus in the semen (370 = 18,000 copies/mL) [37]. Further studies have confirmed that HIV can be intermittently excreted in seminal plasma when serum levels of HIV RNA is undetectable [38–40], and that HIV replication in the genital compartment is promoted by the presence of sexually transmitted diseases (STDs) and other viruses such as cytomegalovirus or herpes simplex. This is why a full sexual health screen is advised prior to offering any form of ART, even in HIV serum viral negative patients on antiretroviral treatment. Rare cases have also been reported where a very high seminal HIV RNA load has been detected despite prolonged use of antiretrovirals and undetectable serum viral load [41–43]. It is deemed highly improbable that these rare cases could lead to sexual viral transmission risk based on the absence of any reported cases of viral transmission through sexual intercourse in HIV-serodiscordant couples when the male is virally fully suppressed through the use of antiretroviral treatment. However, in the IVF laboratory setting, since there is a potential risk of detectable virus being present in semen of a very small percentage of HIV-positive patients on antiretroviral therapy (<5%), best practice dictates that sperm should be processed using a discontinuous density gradient followed by two semen washing steps and then a swim-up. The ESHRE guidance is that the washed sample should then be subjected to an

HIV-PCR, and cites the evidence base for this [2], although the ASRM guidance does not stipulate this PCR step [1, 2, 44]. Sperm washing will of course significantly reduce the quantity of sperm available for treatment and the recommendation that all samples are subjected to a post-wash HIV PCR test will have practical limitations for most centres, necessitating that the sperm sample be electively washed, tested, and frozen ahead of the IVF or ICSI treatment. National guidance should therefore dictate the need for post-wash HIV PCR. Regardless of whether post-wash testing is carried out or not, semen processing should only be carried out by an appropriately trained embryologist, as poor technique has been shown to lead to a higher risk of detectable virus in the post-wash specimen.

In the United States, it has previously been suggested that sperm washing should be combined with ICSI for those wishing to conceive with an HIV-negative female partner [45], irrespective of whether or not semen parameters necessitate this. There is no compelling evidence to support this additional step when semen has been processed on a density gradient followed by two washes and a swim-up, and ICSI should only be offered on the basis of semen parameters not HIV status.

There have been no reported cases of infection of the female partner when sperm washing is carried out following published protocols in more than 11,000 published cycles of sperm washing combined with IUI, IVF, or ICSI [26, 34, 35, 44, 46–51]. ART outcome is reported to compare favourably to that in non-infected patients. A systematic review and meta-analysis of 11,585 cycles of assisted conception with washed sperm in 3994 women reported no single case of HIV transmission [44]. See Table 67.1, reproduced from [44].

Sperm washing has been reported in HIV-positive azoospermic men where testicular sperm retrieval has been performed [52, 53]. The limitations to using a density gradient and two-step washing process are the significant impact on the quantity and quality of sperm available post washing and HIV RNA testing. Thus, in cases necessitating surgical sperm retrieval where sperm count is extremely low, there is a strong argument for ensuring the male has an undetectable serum viral load through effective antiretroviral treatment prior to the procedure to avoid the need for sperm washing post retrieval. ICSI will need to be used due to low sperm numbers and potentially motility.

HIV-positive serodiscordant women

When the female partner is infected with HIV infection and has an undetectable viral load as a result of antiretroviral treatment, she should be encouraged to conceive naturally. As long as the serum viral load remains fully suppressed throughout pregnancy, the risk of mother to child transmission is virtually non-existent. HIV-positive women with fertility issues should be referred for ART and managed in the same way as HIV-negative women. If the woman is not on antiretrovirals for whatever reason, timed self-insemination of her uninfected partner's sperm could be considered. However, it makes no sense to delay the start of antiretroviral therapy when conception is planned, as it should be started once pregnancy is confirmed to minimize the risk of vertical transmission.

HIV-positive women should receive preconceptual counseling by their HIV physician to ensure they are on antiretroviral treatment which is safe in pregnancy and ensure good compliance to reduce mother-to-child transmission (MTCT) risk. They should also review and discuss with the patient any long-term health issues related to HIV which might be a contraindication to

TABLE 67.1 Numbers of Couples and Cycles Reviewed and Number of HIV Seroconversions

Parameter	Result
Initiated cycles of assisted reproduction with washed semen	12,079
Completed cycles of assisted reproduction with washed semen	11,915
Couples with at least one completed cycle of assisted reproduction with washed semen	4257
Women with known HIV results after exposure to washed semen	93.8% (3994/4257)
Completed cycles of assisted reproduction among women with known HIV results after exposure to washed semen	97.2% (11,585/11,915)
Men known to be taking antiretroviral therapy at time of semen washing	39.5% (1685/4257)
Men who were known to have not achieved viral suppression at time of semen washing (plasma testing)	27.7% (985/4257)
Completed cycles of assisted reproduction with the use of washed semen among subgroup of couples with a male partner who was not virally suppressed	24.0% (2863/11,915)
Number of HIV seroconversions (95% CI)	
Per completed cycle of assisted reproduction, overall	0/11,585 (0–0.0001)
Per woman with known HIV outcome, overall	0/3994 (0–0.0004)
Per completed cycle, among subgroup of couples with a male partner who was not virally suppressed	0/2863 (0–0.0006)
Per infant	0/1026 (0–0.0029)

Source: Zafer M et al. *Fertil Steril* 2016;105:645–55.e2, with permission.

Note: CI = confidence interval; HIV = human immunodeficiency virus.

pregnancy. Relatively few antiretroviral medications are contraindicated during pregnancy due to potential teratogenic effects on the fetus but the evidence on the safety of some antiretroviral treatments during pregnancy is under continual review and in some cases incomplete [54]. Folic acid should be given pre-conceptually to HIV-positive women at the same standard dose of 400 µg daily as given to HIV-negative women to minimize the risk of neural tube defects. If co-trimoxazole is being taken, the dose of folic acid should be increased to 5 mg daily.

Guidelines on the management of HIV-positive women in pregnancy, including choice of antiretroviral treatment, vary across different geographical regions.

HIV-concordant couples who are not on antiretroviral treatment

Couples who are both HIV-positive but not on antiretroviral treatment can elect to conceive through timed unprotected intercourse but face the very small risk of superinfection. This is defined as re-infection with a second strain of HIV after the first infection has been established through seroconversion. The true risk is unquantifiable but documented to be very low in patients who are chronically infected with HIV [55]. The risk of superinfection also depends on whether the man and woman are infected with the same HIV strain and whether or not either or both are on antiretroviral treatment. HIV-concordant couples should be

counselled on this risk and both encouraged to start antiretroviral therapy before trying to conceive.

Third-party assisted reproduction (surrogacy) where the intended parent is HIV positive

Professional guidance and national legislation regarding intended parents in surrogacy arrangements where a gestational carrier is involved require, in the vast majority of countries or states, the gamete donor to screen negative for HIV, hepatitis B, and hepatitis C [56]. This excludes those living with HIV from being biological parents in third-party reproductive programmes. In the few geographical areas in the world where it is permissible for the HIV-positive intended parent to be the gamete donor, the HIV status of the gamete donor should be disclosed to the gestational carrier, as well as to other gamete donors involved, such as the egg donor, under the principles of informed consent [56–58]. It is important that the gestational carrier is fully informed of the risks of any such arrangement, and that all measures are taken to minimize the risk to the gestational carrier. The HIV-infected gamete donor should be on antiretroviral therapy and have an undetectable serum viral load, and all steps to reduce viral transmission risk to the gestational carrier should be taken when processing the gametes. Semen from an HIV-positive intended parent should be processed in a density gradient as previously discussed, followed by two washes and a swim-up and post-wash HIV PCR test. The question of whether ICSI should or should not be used is controversial, but, as previously stated, the evidence base for use of ICSI if correct seminal processing has been used is lacking.

Effect of HIV on fertility and ART outcome

In both men and women the effect of HIV and antiretroviral treatment on fertility parameters and ART outcome has been extensively studied.

Effect of HIV on sperm

Studies of HIV-positive men suggest they have semen parameters below the defined WHO normal range. In two large studies of semen parameters in HIV-positive men, Nicopoullos et al. [59, 60] consistently found all parameters to be significantly impaired compared to HIV-negative controls, with a significant negative correlation between CD4 count as a marker of HIV infection and immune status on sperm count, motility, and morphology. There was a significant decrease in volume, count, motility, morphology, and post-wash parameters when CD4 counts dropped below median levels (450 cells/mm^3) but no effect of viral load on any sperm parameter. Similar findings were reported by Kehl [61] with no significant effect of antiretroviral treatment on parameters. Earlier studies [62, 63] by contrast had found viral load to correlate with sperm motility and morphology. A more recent study specifically looking at the effect of antiretroviral treatment on semen parameters found no significant effect on semen parameters other than in those patients on Efarinez where a reduction in sperm motility was noted [64]. The effect of HIV on semen parameters would suggest that HIV-positive men should consider a semen analysis sooner rather than later when attempting to conceive naturally.

A small prospective study has shown antiretroviral therapy to significantly increase sperm DNA integrity in 53 HIV-1 patients receiving antiretroviral treatment (group 1) as compared to 24 naïve HIV-1 patients not receiving antiretroviral treatment (group 2). Increased sperm DNA fragmentation $>30\%$ was demonstrated in 67.9% of patients in group 1 and 37.5% of patients in

group 2, respectively ($p = 0.02$). Increased DNA fragmentation could in turn impact natural fertility and the risk of miscarriage [65].

Effect of HIV on eggs and other female fertility factors

There is increasing evidence to suggest that HIV-positive women have reduced fertility [66–68]. A slight increase in the incidence of cycle irregularity in positive women has been reported, although this dates to a time when antiretroviral treatment was not routinely available [69]. Cycle irregularity was less marked in cases of higher CD4 counts. IVF outcome data has previously suggested that ovarian reserve is reduced in HIV-positive women compared to viral negative women. Antiretroviral treatment may also have a direct effect on oocyte quality by causing mitochondrial toxicity, as mitochondrial depletion has been observed in oocytes of HIV-positive women on antiretroviral therapy [68]. Retrospective data from sub-Saharan Africa [70, 71] and prospective data from the United Kingdom indicates an increased incidence of tubal infertility in positive women [67, 72] of at least twice that of HIV-negative controls. On the basis of increased risk of low ovarian reserve and increased tubal infertility, HIV-positive women trying to conceive should be referred sooner rather than later for fertility evaluation and certainly if they have not conceived within 6 to 12 months. Referral should be earlier if there is a history of pelvic inflammatory disease or in women over 35 in order to assess tubal function and ovarian reserve.

There is no evidence that HIV can attach to or infect oocytes [32] although a single study has reported HIV-1 in follicular fluid and flushes of HIV-positive women undergoing oocyte retrieval for IVF [73]. A study of oocytes from both fresh and frozen cycles has shown increased mitochondrial DNA depletion in women who had been on antiretroviral treatment for more than nine years and had undetectable viral loads [68].

IUI, IVF, and ICSI outcome in HIV-positive patients

The overall conclusion from all studies published to date is that IUI and IVF outcome may be reduced in serodiscordant couples where the female partner is positive, but it should be borne in mind that many of these studies have been conducted over a long time period and do not factor in firstly the impact of co-infection with Hepatitis B and C and secondly the benefit of early antiretroviral intervention, as seen more recently, on long-term general health.

Some early studies suggested reduced ovarian response, implantation, and pregnancy rates in patients with viral infection [72, 74, 75]. A systematic review and meta-analysis in 2011 of serodiscordant couples undergoing ART with sperm washing where the male tested positive for HIV reported on the outcome of 3900 IUI cycles in 1184 couples and 738 IVF/ICSI cycles in 579 couples [51]. The median (range) clinical pregnancy rate was 18% (14.5%–23%) for IUI and 38% (24.8%–46.2%) for IVF. There were no reports of seroconversion of the uninfected female partner or vertical transmission. A later systematic review and meta-analysis in 2014 of 24 studies (including four that were included in Vitorino's study) assessing IUI or IVF outcome in HIV-1 serodiscordant couples measured as primary outcomes HIV transmission risk to the HIV negative partner and per cycle fecundity. Cycle outcome for IUI and IVF for HIV-positive men was 17% and 30%, and for HIV-positive women was 14% and 16%. The study did not compare results with HIV-negative controls. No HIV transmission was noted in 8212 IUI and 1254 IVF cycles [76]. A number of case-controlled studies of HIV-serodiscordant

couples where the male partner is positive have also concluded that a comparable pregnancy rate in ART cycles can be achieved in HIV-serodiscordant couples where sperm washing has been used and that the choice of ART should be determined by the fertility factors not HIV status. A systematic review and meta-analysis of 10 published studies reported on 342 serodiscordant couples where the female partner was HIV-positive. The outcome of 516 IVF/ICSI cycles showed the clinical pregnancy rate per embryo transfer to range from 9.1% to 63%. However a lower pregnancy rate was observed for HIV-positive women in six case-controlled studies, although there was no difference in outcome in four case-controlled studies [77]. The most recent and largest study to date is a retrospective case-controlled study which compared ART outcome in 82 women infected with HIV with outcome in HIV-negative controls. This study found no statistically significant difference between the two groups in response to ovarian stimulation, fertilization rate, or numbers of embryos transferred but a statistically significant lower implantation rate (10% vs 21%), clinical pregnancy rate (12% vs 32%), and live birth rate (7% vs 19%) in HIV-positive women [78] (see Table 67.2). The lower rates have been attributed to a premature fall in ovarian reserve [69] and the impact of antiretroviral treatment on oocyte quality in these women [68].

IVF outcome does not appear to be affected in HIV-positive women undergoing ovum donation, pointing towards an effect of HIV and/or immunosuppression on ovarian response and ovarian reserve rather than on implantation [79].

HIV vertical transmission risk

The use of antiretroviral treatment throughout pregnancy, elective caesarean section only when clinically indicated, and the avoidance of breastfeeding have collectively led to a fall in the MTCT risk from more than 30% to less than 1% [54, 80–82]. Combined neonatal prophylaxis is now recommended for all neonates born to HIV-positive mothers.

Ethical considerations

HIV-infected patients on antiretroviral treatment have a life expectancy approaching that of HIV-negative patients and perinatal vertical transmission risk is approaching zero. These two factors combined have led to widespread acceptance that there are no longer any valid ethical arguments to deny HIV-infected individuals the same reproductive options as viral-negative individuals [58, 82–89]. One exception to this is gamete donation; those living with HIV, hepatitis B or C who are viral load negative as a result of antiretroviral treatment are still excluded in most geographical regions from being able to donate gametes, even

when this is to a known recipient and this applies to third-party reproduction involving surrogacy.

Hepatitis B and C

Hepatitis B (HBV) and hepatitis C (HCV) are major causes of chronic hepatitis, cirrhosis, and hepatocellular cancer.

HBV is a DNA virus and one of the major causes of liver disease worldwide. It can be transmitted sexually, accounting for 40% of transmissions, as well as vertically accounting for another 40% of infections. The sexual transmission risk is twofold higher than for HIV and sixfold higher than for HCV. Overall, as a virus it is about 100 times more infective than HCV or HIV and therefore poses a theoretically higher contamination risk in the laboratory. However, unlike HIV and HCV, an effective vaccine exists for HBV and all healthcare workers and partners of known infected individuals are advised to be vaccinated. Uninfected women with an HBV-infected partner should only consider conception post vaccination. The effectiveness of HBV vaccination is measured by the presence of HBV surface antibody (anti-HBs). Approximately 5% of vaccinated individuals do not produce anti-HBs antibodies and are known as "non-responders." The first step in such cases is to exclude a pre-existing HBV infection by anti-HBc antibody testing. Several options, including combination with hepatitis A vaccination, intradermal application, are available to improve the response in non-infected non-responders [90].

Around 25% of HBV-infected individuals are co-infected with hepatitis-D virus (HDV), which is a replication defective RNA virus that depends on the presence of HBV for replication. Co-infection increases the risk of cirrhosis from 15% to 80%, which is another reason vaccination to eliminate HBV is important as it prevents the transmission of HDV [91].

HBV has been detected in sperm, oocytes, granulosa cells, and embryos. In couples where one or both are infected with HBV, ART clinical protocols should be based on the underlying fertility issues, not HBV status. Priority should always be given to immunizing the uninfected partner first and waiting until anti-HBs antibodies are detected and potentially treating the infected partner with antiretroviral treatment to reduce viral load to undetectable levels before considering assisted conception. Sperm washing has been shown to be ineffective in reducing viral load in HBV-positive serodiscordant males, and is therefore not advised, even when the female partner fails to develop immunity to HBV through vaccination.

Vertical transmission risk for an HBV-positive woman during pregnancy is <10% if she is only HBsAg-positive, and 80%–90% if she is also positive for HBeAg or is HBV DNA positive [92].

TABLE 67.2 Assisted Reproduction Technology (ART) Outcomes in Human Immunodeficiency Virus (HIV)-Infected Women and Matched Controls

ART Outcome	HIV-1 Positive (n = 82), % (n)	HIV Negative Controls (n = 82), % (n)	OR (95% CI) ^a	p-value ^a
Transfer/oocyte retrieval	85 (70/82)	95 (78/82)	0.23 (0.06–0.84)	0.027
Clinical pregnancy/oocyte retrieval	12 (10/82)	32 (26/82)	0.30 (0.13–0.70)	0.006
Clinical pregnancy/embryo transfer	14 (10/70)	33 (26/78)	0.35 (0.15–0.83)	0.017
Implantation rate	10 (10/104)	21 (26/122)	0.38 (0.17–0.87)	0.022
Live birth/embryo transfer	7 (5/70)	19 (15/78)	0.26 (0.08–0.82)	0.022

Source: Stora C, Epelboin S, Devouche E, Matheron S, Epelboin L, Yazbeck C, et al. Women infected with human immunodeficiency virus type I have poorer assisted reproduction outcomes: a case-control study. *Fertil Steril*. 2016;105(5):1193–201, with permission.

In such situations, infection in the neonate can be minimized if immunoprophylaxis (HBV vaccination and one dose of hepatitis B immunoglobulins) is given within 12 hours of birth, with a further dose at one and six months [93]. Breastfeeding does not appear to play a role in perinatal transmission [94]. However, this approach is not sufficient to prevent vertical transmission if the HBV DNA level in the mother exceeds 200,000 IU/mL. Therefore, current management is to consider antiviral therapy of the pregnant woman if HBV DNA load exceeds 200,000 IU/mL. Breastfeeding is not contraindicated in mothers who have chronic HBV infection, nor is it contraindicated in those who are on antiretroviral treatment, as these are minimally excreted in breast milk.

HCV infection is primarily transmitted by parenteral spread (blood products, shared needles, needle-stick injury). Although sexual transmission has been observed in HIV-positive men who have sex with men [95], the transmission appears to be limited to specific sexual practices involving exchange of blood [96]. Heterosexual transmission among monogamous HCV-discordant partners is essentially non-existent and the use of condoms is not recommended for these couples. Natural conception is advised, but if fertility issues exist, ART should be offered based on fertility factors. When the male is HCV positive, the issue of whether sperm washing should be performed is controversial. Earlier studies advised against sperm washing [97, 98], but a careful review of all studies to date would indicate that HCV can still be detected following a single discontinuous density gradient and a swim-up, and a further washing step is therefore necessary. PCR testing of the post-wash sample is not deemed necessary, as viral loads in semen are very low and HCV has not been detected in post-wash semen samples [2, 99].

There is currently no vaccine for HCV but prior to ART, antiviral treatment is advised for those infected individuals, male and female, who meet the country-specific requirements for HCV treatment and should be offered prior to planning conception with the aim of clearing the virus. HCV is treated with direct-acting antiviral therapy, which is 98% effective in achieving a sustained virologic response as measured by a negative HCV RNA within 12 weeks of starting treatment [57]. Direct-acting anti-retrovirals are teratogenic and none are licensed to be used in pregnancy. The decision to treat is normally taken in conjunction with a patient's infectious disease or hepatology expert and must take into consideration the risks of delaying conception against the benefits of reducing viral transmission risks during conception and pregnancy and improving the health of the individual.

Vertical transmission risk in HCV-RNA positive women is around 5%–6% and doubles for HIV-positive mothers [100]. For HCV-RNA and HIV-negative mothers, vertical transmission of HCV has not been observed [101–103]. In the absence of a vaccine, there are no specific measures available to protect the neonate and the administration of immunoglobulin offers no protection. There are no data to suggest HCV is transmitted during breastfeeding and no indications for caesarean section delivery [102].

ART outcome for patients infected with HBV and HCV

Recent studies on HBV-positive men and women suggest overall no significant difference in outcome with ART compared to non-infected controls [104, 105]. In the case of males infected with HCV, some studies report a significant negative effect of HCV on semen quality [106] and fertilization rates, and other studies show no significant difference in assisted reproductive outcome

in these couples. There are also conflicting results in HCV-positive women, with some studies reporting lower implantation rates, higher cycle cancellation rates, and higher doses of FSH, and others showing no significant differences in these variables compared to HCV-negative controls [2, 107, 108].

In the case of men infected with HBV or HCV where sperm washing is used in IVF/ICSI there are no reported differences in cycle outcome compared to non-infected cases.

Human T-lymphotropic virus I and II

Whilst the majority of scientific publications on viral infection in the ART setting focus on HIV and hepatitis, consideration should also be given to human T-lymphotropic virus type 1 and 2 infection (HTLV-1/2). This is an ancient retrovirus that infects CD4 T cells. Although transmitted vertically, intravenously, and by sexual contact, the modes of transmission are distinct from HIV and hepatitis: vertical transmission occurs primarily through breastfeeding. Sexual transmission is much more efficient from male to female (60% in a 10-year partnership) than the opposite direction (<1% in 10 years) [109]. The potential for causing human disease is far lower than HIV, HBV, and HCV, but 1%–4% of infected patients develop adult T-cell leukaemia or spastic paraparesis. Low endemic rates (1%) are reported in North and South America, Africa, and Japan. Studies of prevalence in the assisted reproduction population in Sweden suggest a seroprevalence of 2.3 per 10,000 [110].

Screening for HTLV-1/2 In all women seeking ART services is not required, but targeted screening of individuals from endemic areas (Caribbean, South America, Central Africa, Japan) should be considered [2, 111], particularly if they are to be gamete donors. Couples where one partner is found to be HTLV-1/2 positive should be counselled as outlined in a CDC recommendation [112]. Given the lack of a recommendation for barrier precautions in HTLV-1/2 serodiscordant couples, natural conception is the preferred mode of conception. ART in infertile couples affected by HTLV-1/2 should only be performed if fertility factors exist and with the standard precautions when handling biologic materials. There have been no studies assessing the impact of semen washing on reducing horizontal transmission risk and it is currently not recommended [2].

There are no reliable data to suggest that women infected with HTLV-1/2 should be delivered by caesarean section to reduce vertical transmission risk and the increased risk of an operative delivery cannot therefore be justified. However, they should be advised against breastfeeding their infants, as there are a number of good studies to show this reduces mother-to-child transmission risk [2, 113, 114].

Zika virus

Zika virus (ZIKV) is a mosquito-transmitted single-stranded RNA flavivirus. It was first identified and isolated from mosquitos from the Zika forest in Uganda in 1947. The incubation period for the ZIKV is up to two weeks, and the majority of cases are asymptomatic with symptoms only seen in 20% of those infected. Symptoms include fever, myalgia, conjunctivitis, and rash. Although the main form of viral transmission is by mosquitos, sexual transmission of Zika is increasingly reported [115, 116], as is transmission through blood transfusion. RNA Zika virus has been detected in both semen and vaginal fluid [117, 118]. Following the recent epidemic reported in 2015–16, ZIKV has

been linked to congenital Zika syndrome, which consist of multiple developmental issues including microcephaly and fetal loss. If ZIKV is contracted in the first trimester of pregnancy, the risk of fetal neurological damage is as high as 10% [119, 120]. ZIKV has also been associated with Guillain-Barre syndrome [121].

An early study of Zika RNA in blood, urine, and semen from a 32-year-old man returning from French Guyana showed persistence of the virus in semen for up to 141 days after onset of symptoms but no detectable virus in the plasma or urine from 37 days after onset of symptoms (Figure 67.1 reproduced from reference [118]). A number of further reports have confirmed variable persistence of the virus in semen after initial infection [116, 118, 122, 123], which has led to ZIKV being classified as a virus of concern in all those attempting to conceive naturally or through ART who have recently visited or live in an area where Zika is endemic. When counselling patients planning to conceive, relevant professionals should refer to the WHO website, which publishes regular updated global epidemiological surveillance data on Zika transmission risk, congenital Zika syndrome, and relevant data on countries where ZIKV is endemic (<https://www.who.int/publications/m/item/zika-epidemiology-update—february-2022>). Current data indicates ZIKV is prevalent in South and Central America, the Caribbean, the Pacific Islands, Africa, India, and the Far East. Guidance on minimizing transmission risk in the ART setting has also been published by the CDC (<https://www.cdc.gov/zika/>), FDA, ESHRE [2], and ARSM, whose latest guidance was published in 2019 and based on CDC, FDA, and WHO published guidance. Where it is available, local guidance should provide an additional reference point. Currently, no vaccine or treatment exists to prevent Zika virus syndrome.

ZIKV nucleic acid testing (NAT) is the most sensitive test for early detection of ZIKV (recently infected or presenting with onset of symptoms with the previous seven days) but as ZIKV levels drop significantly after seven days after the onset of infection, these may not be detected by the NAT test and serological-based tests such as immunoassays and immunofluorescence assays to detect IgM should be used. However, ZIKV IgM can persist for years and there is cross reactivity between ZIKV and Dengue IgM antibodies in serological testing, as Dengue virus is another flavivirus.

Men diagnosed with ZIKV infection or returning from a ZIKV endemic region should use barrier contraception with any partner for three months to avoid horizontal transmission, and their female partners should avoid pregnancy for two months to avoid vertical transmission [2]. Females diagnosed with ZIKV infection or returning from a ZIKV endemic region are advised by the CDC to avoid a pregnancy for eight weeks, although the WHO advises to avoid pregnancy for six months. ESHRE guidance advises two months [2]. If a patient or their partner is diagnosed with ZIKV or is returning from a ZIKV endemic region they are advised to postpone ART. If diagnosed during the cycle, treatment should be stopped.

There is as yet very little data to demonstrate the integration of ZIKV in either eggs or sperm [124, 125]. Careful counselling regarding such potential risks should be provided in those needing to undergo urgent fertility preservation of gametes or embryos if they are known to be infected with ZIKV [2].

There are no specific semen washing techniques that can remove ZIKV from semen [125] and no reliability should be placed on measuring serum ZIKV load due to the poor correlation between serum and semen ZIKV levels [2].

An alternative for asymptomatic men and women undergoing ART who either live or have recently returned from a ZIKV endemic region is to consider NAT testing for ZIKV infection prior to starting the ART cycle although this is only sensitive within the first seven days of infection.

For women diagnosed with ZIKV during pregnancy, there is no evidence that viral transmission to the new-born is reduced by either caesarean section or avoidance of breastfeeding.

Covid-19 (SARS-CoV-2)

In December 2019, a cluster of cases of pneumonia of unknown cause was reported in Wuhan City, Hubei Province, China. A novel coronavirus named Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) was subsequently identified from patient samples. This is of zoonotic origin, but the source of the outbreak has never been determined. The matter was reported to the WHO and the rapid rise in cases internationally in the ensuing months led to the WHO declaring Coronavirus-19 (Covid-19)

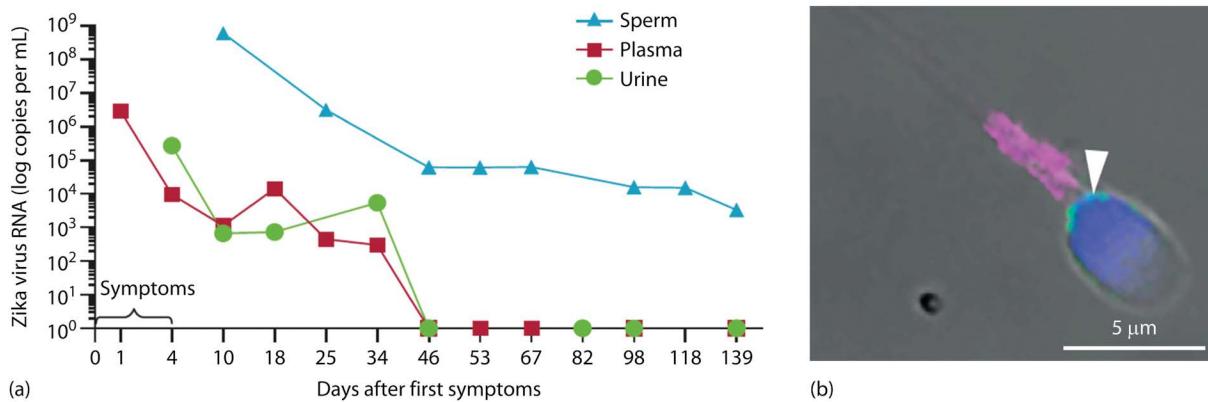


FIGURE 67.1 Zika virus infects spermatozoa. (a) The kinetics of Zika virus RNA detection in plasma, urine, and semen quantified by real-time polymerase chain reaction (RealStar Zika Virus RT-PCR Kit 1.0, Altona Diagnostics GmbH, Hamburg, Germany). (b) Immunohistochemical detection of Zika virus (green; arrowhead) on bright-field microscopy in the head of spermatozoa obtained from a patient; Tom20 is pink, Zika virus is green, and Hoechst stain is blue. (From Mansuy JM et al. Lancet Infect Dis 2016;16:405, with permission.)

a global pandemic. SARS-CoV-2 is primarily transmitted by respiratory droplets and aerosol-generating procedures (AGPs) releasing infected particles from the respiratory tract. Transmission risk is increased by close proximity to an infected person, and good ventilation in indoor settings, use of face masks, and avoidance of AGPs have been recommended in the healthcare setting to reduce transmission risk. Fomite transmission is a lower risk but has been reported as a result of contact with surfaces contaminated with infected particles. This risk can be reduced in the healthcare setting with regular cleaning of exposed surfaces with appropriate antiseptic wipes.

Whilst Covid-19 was noted to cause relatively mild symptoms in many of those infected, including a high temperature, cough, and loss or change to smell or taste, 15% of those infected developed more severe symptoms, leading to hospitalization, oxygen support, and ventilation, and 5% had respiratory failure, acute respiratory distress syndrome, sepsis, and multiorgan failure leading to concerning fatality rates being reported in the early months of the pandemic. Trigger factors for more severe disease include obesity, older age, reduced immunity, and ethnicity. An effective vaccine is now available and many variants now reported are milder, leading to significantly lower fatality rates.

The mean incubation period for SARS-CoV-2 varies, with the variant ranging from three to six days. Contact tracing has shown that the highest risk of infection occurs a few days before symptom onset to a few days after, and that by 10 days after symptom onset infectiousness is low.

Testing for SARS-CoV-2 is now readily available through the rapid lateral flow test, which is a viral antigen based immunoassay. These home tests providing results with 10 minutes and are a useful screening tool in the ART setting for both patients and staff to identify risk in patients who are attending the centre for treatment but may be asymptomatic.

Since 2020, much attention has since been placed on collecting data on the impact of on fertility, pregnancy, and ART outcome. As new guidance becomes available and a transition to an endemic status becomes more likely, professionals are advised to refer to the latest available guidance when planning services and counselling patients. In addition, new variants are constantly emerging and these may have a less-severe impact on general as well as fertility health in both the non-pregnant and pregnant state. The latest available guidance from the ASRM, ESHRE, and UK-based Association of Reproductive and Clinical Scientists and British Fertility Society have common recommendations, which are detailed in this chapter, but updated guidance should always be followed.

All patients considering conception whether naturally or through ART are advised to receive Covid-19 vaccination and complete the required programme where available. Numerous studies have shown that Covid-19 in pregnancy is associated with much higher risk of needing ventilation and intensive care support than in non-pregnant controls. There is also an increased risk to the fetus of low birth weight, premature delivery, and stillbirth [126, 127]. The majority of hospital admissions in pregnant women with Covid-19 are in those who have not been vaccinated. There is also an increased risk of first trimester miscarriage in women infected with SARS-CoV-2 [128].

There is reassuring evidence to show the vaccine does not negatively impact fertility health and has no impact on sperm parameters. By contrast, infection with SARS-CoV-2 does negatively affect semen parameters and can, in some cases, dysregulate endocrine function [129], but there is no evidence it affects

ovarian reserve [130–133], despite the transient variations in menstrual cycle regularity which have been reported both after Covid-19 infection and the vaccine [134]. The vaccine has been shown to be safe even when given in early pregnancy [135] and is very effective at reducing the severity of the disease.

SARS-CoV-2 can enter the testes but not the ovary. It can also enter human embryo cells through the trophectoderm. Studies looking at IVF outcome in patients recovering from Covid-19 are limited and numbers are too small to draw solid conclusions. Orvieto carried out an observational study on nine couples, seven females and two males with cycles before and after Covid-19 infection and found no overall differences in cycle outcome but a reduced number of top-quality embryos to transfer or cryopreserve. Wang et al. reported on 70 females undergoing ART who tested positive for SARS-CoV-2 compared to 3973 who tested negative. No differences were observed in ovarian response, proportion of mature oocytes, fertilization rates, cleavage rates, numbers of top-quality blastocysts, clinical pregnancy rates, and miscarriage rates [136]. However, since infection has been shown to negatively impact semen parameters [129], it is currently recommended that infected male patients delay ART for three months following infection with SARS-CoV-2. Any delay to treatment should also consider the severity of the infection, as symptoms of long Covid in an affected female partner could potentially impair response and outcome to ART.

Patients who test positive for SARS-CoV-2 during treatment are advised to cease treatment to minimize the risk of infection to other patients and staff.

Monkeypox

Monkeypox is caused by the MPXV and causes a viral zoonotic disease that is primarily found in the tropical rainforests areas of Central and West Africa. The DNA virus is related to small pox but is less contagious or severe. It causes a short-lived viral illness including fever, a characteristic rash and swollen lymph nodes. Symptoms last 2 to 4 weeks although more severe cases leading to death have been reported in 3%–6% of patients. An antiviral agent is available. Person to person transmission occurs through body fluids including semen and respiratory droplets.

In 2022 an outbreak of Monkey pox was reported. Concern has been expressed about the risk to recipients of substances of human origin such as blood and tissues and therefore includes partner and donor sperm and eggs. The risk of viral transmission in the ART setting is primarily linked to provision of semen by an infected male partner. No screening guidance has yet been released with regards to sperm and egg donation in the ART setting but until such guidance is published, affected individuals are advised not to donate.

The epidemiology suggests highest frequency is in individuals with multiple sexual partners and in men having sex with men. As this is a relatively new virus to deal with in the assisted conception setting, the latest data should be sought from WHO (<https://www.who.int/news-room/fact-sheets/detail/monkeypox>), CDC (<https://www.cdc.gov/poxvirus/mpox/index.html>) and European Centre for Disease Control (<https://www.ecdc.europa.eu/en/mpox-monkeypox>).

MPXVDNA has been detected in the semen of men up to 11 days after acute infection. A recent meta-analysis has found the pooled prevalence of MPXR DNA to be 72.4% among 115 patients from 5 eligible studies. The infectivity of MPXV

in semen was also proven by demonstrating their replication potential in two out of four patients (see reference to add here in attached email). Current WHO and UK guidance is that men who have been infected with Monkeypox should use condoms during sex and not donate sperm until they have been shown to test negative. In cases where the patient is planning fertility treatment, semen storage or has an immunocompromised partner including a female partner who is pregnant, a monkeypox PCR can be performed on semen samples from as early as day 1 and up to day 19 after initial Monkeypox infection. A single negative PCR test is considered adequate.

Viral screening prior to offering assisted conception

The majority of ART centres globally require patients presenting for ART to be screened for HIV-1 and 2, HBV, and HCV. Certain groups of patients will need to be screened for HTLV-1/2 if they have travelled or work in high-risk areas where the virus is endemic. This has the benefit of identifying high-risk patients from the outset so that they can receive appropriate counselling and start antiretroviral treatment before embarking on conception. National guidelines on screening frequency and timing in relation to the ART procedure vary widely. All gamete donors are required to be screened and, in the majority of countries or states, viral positive patients are excluded from donating. In the case of sperm donors, full viral and sexual health screening is required before semen collection for cryo-storage commences, as well as after a quarantine period once all the samples of sperm have been collected and frozen. Use of RNA NAT testing has the benefit of allowing for a shorter three-month quarantine period post storage as opposed to six months if routine serology antibody testing is used.

In the case of egg donors, the situation is more complex and policy on timing of screening varies in different countries. Ideally egg donors should be screened as closely as possible to the timing of vaginal egg collection to minimize any risk. In practice most national guidelines advise screening as egg donors start ovarian stimulation.

Laboratory protocols for viral positive patients

Despite previously published concerns regarding the handling and freezing of gametes and embryos from patients who carry bloodborne viruses [137–139], there are no reported cases of cross-contamination in the ART setting. Published guidelines on the matter are limited [2, 57, 137], and many ART centres still do not treat patients with a known viral infection. ESHRE guidance in 2008 recommended that gametes and embryos from patients infected with HIV, HBV, and HCV be handled in a dedicated laboratory space at allocated times and processing of these samples within a biosafety cabinet to minimize the risk of cross-contamination of patient specimens. The use of separate labs is not advised in their most recent guidance of 2022, as there are no published studies to support such practice, in contrast to the most recent guidance from ASRM which still recommends processing of samples from viral positive patients in a separate laboratory [2]. ART centres should therefore base their approach on their own country's legislation as well as their own risk assessment of their service. In addition to using universal precautions in the assisted conception laboratory, best practice dictates that samples from

patients with known or suspected bloodborne viruses should ideally be handled separately in time or space to reassure viral-negative patients that all measures have been taken to minimize any risk of viral contamination. In addition, single-use devices should always be considered where possible.

There is wide variation between countries when it comes to cryo-storage of gametes and embryos from patients with known viral infections. Latest ESHRE and ASRM published guidance recommend these are cryopreserved using closed-system vitrification systems such as heat-sealed straws [140] and in separate cryo-storage tanks due to a theoretical risk of transmission in liquid nitrogen [141, 142]. Although it has been suggested that vapour phase storage would offer more security against the risk of cross-contamination as compared to liquid nitrogen without affecting embryo and sperm survival [143], there are no long-term data to assess safety and efficacy of this approach.

What is clear from reviewing the published literature is that there are no universally agreed guidelines and local policies should determine laboratory practice. The emergence of new viruses such as the SAR-CoV-2 and MPXV exemplifies the importance of treating all samples as potentially infectious and using universal precautions at all times, as is used in operating theatres and emergency rooms to ensure there is no difference between handling a sample from a known virally infected patient and a sample from a patient who has screened negative for those viruses routinely screened for, i.e. HIV, HBV, and HCV. There is certainly no longer any ethical justification for denying a viral positive patient assisted conception on the grounds of inadequate laboratory facilities if universal precautions and the aforementioned measures can be applied.

Conclusion

No ART centre should find itself unable to offer a viral positive patient treatment other than on ethical grounds unrelated to viral infection.

Antiretroviral therapy has radically changed the natural course of HIV infection and in turn the way professionals should manage reproductive care for these patients. Those individuals who as a result of treatment become viral negative can expect to lead near normal lives and have children naturally through unprotected intercourse. Where fertility issues exist, these patients should be offered the same, full spectrum of assisted conception treatments offered to non-infected individuals within a safe laboratory environment equipped to deal with both known and unknown viral risk.

Patients infected with HBV and HCV should be offered very similar guidance to those with HIV, and, in the case of HBV, vaccination should be offered to the uninfected partner.

The emergence of newer viruses of concern including ZIKV, SARS-CoV-2 and MPXV demonstrate the need for vigilance in the ART setting, for professionals to make continued reference to updated evidence-based guidance on these viruses and the use of universal precautions in the laboratory setting.

Reproductive counselling for patients with a known viral infection should only be offered by appropriately qualified personnel able to discuss viral transmission risk in both natural and assisted conception settings and the effects of the virus and/or the antiviral treatment on fertility, as well as horizontal and vertical transmission risk so as to fully inform these patients of their risks and options. Reproductive specialists and patients share the responsibility of preventing viral infection to the uninfected partner and child as to other patients and staff attending the centre.

References

1. Ethics Committee of the American Society for Reproductive Medicine. Human immunodeficiency virus and infertility treatment: An ethics committee opinion. *Fertil Steril.* 2021;115(4):860–9.
2. ESHRE Guideline Group on Viral infection/disease, Mocanu E, Drakeley A, Kupka MS, Lara-Molina EE, Le Clef N, et al. ESHRE guideline: Medically assisted reproduction in patients with a viral infection/disease. *Hum Reprod Open.* 2021;2021(4):hoab037.
3. Doitsh G, Galloway NL, Geng X, Yang Z, Monroe KM, Zepeda O, et al. Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. *Nature.* 2014;505(7484):509–14.
4. Schnittman SM, Fauci AS. Human immunodeficiency virus and acquired immunodeficiency syndrome: An update. *Adv Intern Med.* 1994;39:305–55.
5. Hart C, Schochetman G, Spira T, Lifson A, Moore J, Galpin J, et al. Direct detection of HIV RNA expression in seropositive subjects. *Lancet.* 1988;2(8611):596–9.
6. Branson BM. HIV diagnostics: Current recommendations and opportunities for improvement. *Infect Dis Clin North Am.* 2019;33(3):611–28.
7. Parekh BS, Ou CY, Fonjungo PN, Kalou MB, Rottinghaus E, Puren A, et al. Diagnosis of human immunodeficiency virus infection. *Clin Microbiol Rev.* 2019;32(1):e00064-18.
8. Hogg RS, Heath KV, Yip B, Craib KJ, O'Shaughnessy MV, Schechter MT, et al. Improved survival among HIV-infected individuals following initiation of antiretroviral therapy. *Jama.* 1998;279(6):450–4.
9. Nakagawa F, May M, Phillips A. Life expectancy living with HIV: Recent estimates and future implications. *Curr Opin Infect Dis.* 2013;26(1):17–25.
10. van Sighem AI, Gras LA, Reiss P, Brinkman K, de Wolf F. Life expectancy of recently diagnosed asymptomatic HIV-infected patients approaches that of uninfected individuals. *AIDS.* 2010;24(10):1527–35.
11. Scandlyn J. When AIDS became a chronic disease. *West J Med.* 2000;172(2):130–3.
12. Vernazza P, Hirscher B, Bernasconi E, Flepp M. Les personnes séropositives ne souffrant d'aucune autre MST et suivant un traitement antirétroviral efficace ne transmettent pas le VIH par voie sexuelle. *Bulletin des Médecins Suisses.* 2008;89(5):165–9.
13. Vernazza P, Bernard EJ. HIV is not transmitted under fully suppressive therapy: The Swiss Statement—eight years later. *Swiss Med Wkly.* 2016;146:w14246.
14. Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, et al. Antiretroviral therapy for the prevention of HIV-1 transmission. *N Engl J Med.* 2016;375(9):830–9.
15. Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, et al. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med.* 2011;365(6):493–505.
16. Rodger AJ, Cambiano V, Bruun T, Vernazza P, Collins S, Degen O, et al. Risk of HIV transmission through condomless sex in serodifferent gay couples with the HIV-positive partner taking suppressive antiretroviral therapy (PARTNER): Final results of a multicentre, prospective, observational study. *Lancet.* 2019;393(10189):2428–38.
17. LeMessurier J, Traversy G, Varsaneux O, Weekes M, Avey MT, Niragira O, et al. Risk of sexual transmission of human immunodeficiency virus with antiretroviral therapy, suppressed viral load and condom use: A systematic review. *CMAJ.* 2018;190(46):E1350–E60.
18. Bujan L, Pasquier C. People living with HIV and procreation: 30 years of progress from prohibition to freedom? *Hum Reprod.* 2016;31(5):918–25.
19. Vandermaelen A, Englert Y. Human immunodeficiency virus serodiscordant couples on highly active antiretroviral therapies with undetectable viral load: Conception by unprotected sexual intercourse or by assisted reproduction techniques? *Hum Reprod.* 2010;25(2):374–9.
20. Vernazza PL, Graf I, Sonnenberg-Schwan U, Geit M, Meurer A. Preexposure prophylaxis and timed intercourse for HIV-discordant couples willing to conceive a child. *AIDS.* 2011;25(16):2005–8.
21. Mujugira A, Celum C, Coombs RW, Campbell JD, Ndase P, Ronald A, et al. HIV transmission risk persists during the first 6 months of antiretroviral therapy. *J Acquir Immune Defic Syndr.* 2016;72(5):579–84.
22. Anglemeyer A, Rutherford GW, Baggaley RC, Egger M, Siegfried N. Antiretroviral therapy for prevention of HIV transmission in HIV-discordant couples. *Cochrane Database Syst Rev.* 2011(8):Cd009153.
23. Whetham J, Taylor S, Charlwood L, Keith T, Howell R, McInnes C, et al. Pre-exposure prophylaxis for conception (PrEP-c) as a risk reduction strategy in HIV-positive men and HIV-negative women in the UK. *AIDS Care.* 2014;26(3):332–6.
24. Hoffman RM, Jaycox A, Vardavas R, Wagner G, Lake JE, Mindry D, et al. Benefits of PrEP as an adjunctive method of HIV prevention during attempted conception between HIV-uninfected women and HIV-infected male partners. *J Infect Dis.* 2015;212(10):1534–43.
25. Sun L, Wang F, Liu A, Xin R, Zhu Y, Li J, et al. Natural conception may be an acceptable option in HIV-serodiscordant couples in Resource limited settings. *PLoS One.* 2015;10(11):e0142085.
26. Semprini AE, Levi-Setti P, Bozzo M, et al. Insemination of HIV-negative women with processed semen of HIV-positive partners. *Lancet.* 1992;340:1317–9.
27. Bagasra O, Farzadegan H, Sesamma T, et al. Detection of HIV-1 proviral DNA in sperm from HIV-1 infected men. *AIDS.* 1994;8:1669–74.
28. Vernazza PL, Gilliam BL, Dyer J, et al. Quantification of HIV in semen: Correlation with antiviral treatment and immune status. *AIDS.* 1997;11:987–93.
29. Quayle AJ, Xu C, Mayer KH, Anderson DJ. T lymphocytes and macrophages, but not motile spermatozoa, are a significant source of human immunodeficiency virus in semen. *J Infect Dis.* 1997;176:960–8.
30. Quayle AJ, Xu C, Tucker L, Anderson DJ. The case against an association between HIV-1 and sperm: Molecular evidence. *J Reprod Immunology.* 1998;41:127–36.
31. Kim LU, Johnson MR, Barton S, Nelson MR, Sontag G, Smith JR, et al. Evaluation of sperm washing as a potential method of reducing HIV transmission in HIV-discordant couples wishing to have children. *AIDS.* 1999;13(6):645–51.
32. Baccetti B, Benedetto A, Burrini AG, et al. HIV particles detected in spermatozoa of patients with AIDS. *J Submicrosc Cytol Pathol.* 1991;23:339–45.
33. Politch JA, et al. Separation of human immunodeficiency virus type 1 from motile sperm by the double tube gradient method versus other methods. *Fertil Steril.* 2004;81(2):440–7.
34. Marina S, Marina F, Alcolea R, et al. Pregnancy following intracytoplasmic sperm injection from an HIV-1 seropositive man. *Hum Reprod.* 1998;13:3247–9.
35. Marina S, Marina F, Alcolea A, et al. Human immunodeficiency virus type I-serodiscordant couples can bear healthy children after undergoing intrauterine insemination. *Fertil Steril.* 1998;70:35–9.
36. Gilling-Smith C, Nicopoulos JDM, Semprini AE, Frodsham LCG. HIV and reproductive care - a review of current practice. *BJOG.* 2006;113:1–10.
37. Vourliotis M, Nicopoulos JDM, Gilling-Smith C, Andronikou S, Almeida P, Williamson P, Almeida A comparison of sperm yield following changes in HIV sperm washing laboratory practice. *Hum Reprod.* 2009;24(Supplement 1):i166.
38. Vernazza PL, Gilliam BL, Flepp M, Dyer JR, Frank AC, Fiscus SA, et al. Effect of antiviral treatment on the shedding of HIV-1 in semen. *AIDS.* 1997;11(10):1249–54.
39. Vernazza PL, Troiani L, Flepp MJ, Cone RW, Schock J, Roth F, et al. Potent antiretroviral treatment of HIV-infection results in suppression of the seminal shedding of HIV. The Swiss HIV cohort study. *AIDS.* 2000;14(2):117–21.

40. Leruez-Ville M, Dulouste E, Costabliola D, Salmon D, Tachet A, Finkelsztejn L, et al. Decrease in HIV-1 seminal shedding in men receiving highly active Antiretroviral therapy: An 18 month longitudinal study (AnRS EP012). *AIDS*. 2002;16(3):486–8.
41. Pasquier CJ, Moinard N, Saune K, Souyris C, Lavit M, Daudin M, et al. Persistent differences in the antiviral effects of highly active antiretroviral therapy in the blood and male genital tract. *AIDS*. 2008;22(14):1894–6.
42. Pasquier C, Moinard N, Saune K, Daudin M, Trancart S, Massip P, et al. Antiviral effect of maraviroc in semen: A case report. *Antivir Ther*. 2012;17(5):933–6.
43. Pasquier C, Walschaerts M, Raymond S, Moinard N, Saune K, Daudin M, et al. Patterns of residual HIV-1 RNA shedding in the seminal plasma of patients on effective antiretroviral therapy. *Basic Clin Androl*. 2017;27:17.
44. Zafer M, Horvath H, Mmeje O, van der Poel S, Semprini AE, Rutherford G, et al. Effectiveness of semen washing to prevent human immunodeficiency virus (HIV) transmission and assist pregnancy in HIV-discordant couples: A systematic review and meta-analysis. *Fertil Steril*. 2016;105(3):645–55.e2.
45. Jindal SK, Rawlins RG, Muller CH, Drobis EZ. Guidelines for risk reduction when handling gametes from infectious patients seeking assisted reproductive technologies. *Reprod Biomed Online*. 2016;33(2):121–30.
46. Ohl JP, Wittemer M C, et al. Assisted reproduction techniques for HIV serodiscordant couples: 18 months of experience. *Hum Reprod*. 2003;18:1244–9.
47. Garrido N, Meseguer M, Simon C, Pellicer A, Remohi J. Assisted reproduction in HIV and HCV infected men of serodiscordant couples. *Arch Androl*. 2004;50(2):105–11.
48. Bujan L, Hollander L, Coudert M, Gilling-Smith C, Vucetich A, Guibert J, et al. Safety and efficacy of sperm washing in HIV-1-serodiscordant couples where the male is infected: Results from the European CREATHE network. *AIDS*. 2007;21(14):1909–14.
49. Sauer MV, Wang JG, Douglas NC, Nakhuda GS, Vardhana P, Jovanovic V, et al. Providing fertility care to men seropositive for human immunodeficiency virus: Reviewing 10 years of experience and 420 consecutive cycles of in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril*. 2009;91(6):2455–60.
50. Nicopoullos JD, Almeida P, Vourliotis M, Goulding R, Gilling-Smith C. A decade of sperm washing: Clinical correlates of successful insemination outcome. *Hum Reprod*. 2010;25(8):1869–76.
51. Vitorino RL, Grinsztejn BG, de Andrade CA, Hokerberg YH, de Souza CT, Friedman RK, et al. Systematic review of the effectiveness and safety of assisted reproduction techniques in couples serodiscordant for human immunodeficiency virus where the man is positive. *Fertil Steril*. 2011;95(5):1684–90.
52. Nicopoullos JD, Frodsham LC, Ramsay JW, Almeida PA, Rozis G, Gilling-Smith C. Synchronous sperm retrieval and sperm washing in an intracytoplasmic sperm injection cycle in an azoospermic man who was positive for human immunodeficiency virus. *Fertil Steril*. 2004;81(3):670–4.
53. Bujan L, Daudin M, Moinard N, Plante P, Parinaud J, Pasquier C. Azoospermic HIV-1 infected patients wishing to have children: Proposed strategy to reduce HIV-1 transmission risk during sperm retrieval and intracytoplasmic sperm injection: Case report. *Hum Reprod*. 2007;22(9):2377–81.
54. Gilleece DY, Tariq DS, Bamford DA, Bhagani DS, Byrne DL, Clarke DE, et al. British HIV association guidelines for the management of HIV in pregnancy and postpartum 2018. *HIV Med*. 2019;20(Suppl 3):s2–85.
55. Fakoya A, Lamba H, Mackie N, Nandwani R, Brown A, Bernard E, et al. British HIV association, BASHH and FSRH guidelines for the management of the sexual and reproductive health of people living with HIV infection 2008. *HIV Med*. 2008;9(9):681–720.
56. Practice Committee of the American Society for Reproductive Medicine and Practice Committee of the Society for Assisted Reproductive Technology. Recommendations for practices utilizing gestational carriers: A committee opinion. *Fertil Steril*. 2015;103(1):e1–8.
57. Ethics Committee of the American Society for Reproductive Medicine. Interests, obligations, and rights in gamete and embryo donation: An ethics committee opinion. *Fertil Steril*. 2019;111(4):664–70.
58. Ethics Committee of American Society for Reproductive Medicine. Human immunodeficiency virus (HIV) and infertility treatment: A committee opinion. *Fertil Steril*. 2015;104(1):e1–8.
59. Nicopoullos JD, Almeida PA, Ramsay JW, Gilling-Smith C. The effect of human immunodeficiency virus on sperm parameters and the outcome of intrauterine insemination following sperm washing. *Hum Reprod*. 2004;19(10):2289–97.
60. Nicopoullos JD, Almeida P, Vourliotis M, Gilling-Smith C. A decade of the sperm-washing programme: Correlation between markers of HIV and seminal parameters. *HIV Medicine*. 2011;12(4):195–201.
61. Kehl S, Weigel M, Muller D, Gentili M, Hornemann A, Sutterlin M. HIV-infection and modern antiretroviral therapy impair sperm quality. *Arch Gynecol Obstetr*. 2011;284(1):229–33.
62. Dulouste E, Du AL, Costagliola D, Guibert J, Kunstmann JM, Heard I, et al. Semen alterations in HIV-1 infected men. *Hum Reprod*. 2002;17(8):2112–8.
63. Bujan L, Sergerie M, Moinard N, Martinet S, Porte L, Massip P, et al. Decreased semen volume and spermatozoa motility in HIV-1-infected patients under antiretroviral treatment. *J Androl*. 2007;28(3):444–52.
64. Frapsauce C, Grabar S, Leruez-Ville M, Launay O, Sogni P, Gayet V, et al. Impaired sperm motility in HIV-infected men: An unexpected adverse effect of efavirenz? *Hum Reprod*. 2015;30(8):1797–806.
65. Savasi V, Oneta M, Laoreti A, Parisi F, Parrilla B, Duca P, et al. Effects of antiretroviral therapy on sperm DNA integrity of HIV-1-infected men. *Am J Mens Health*. 2018;12(6):1835–42.
66. Coll O, Lopez M, Vidal R, Figueras F, Suy A, Hernandez S, et al. Fertility assessment in non-infertile HIV-infected women and their partners. *Reprod Biomed Online*. 2007;14(4):488–94.
67. Frodsham LCGBF, Barton S, Gilling-Smith C. Human immunodeficiency virus infection and fertility care in the United Kingdom – Demand and supply. *Fert Steril*. 2006;85:285–9.
68. Lopez S, Coll O, Durban M, Hernandez S, Vidal R, Suy A, et al. Mitochondrial DNA depletion in oocytes of HIV-infected anti-retroviral-treated infertile women. *Antivir Ther*. 2008;13(6):833–8.
69. Massad LS, Evans CT, Minkoff H, Watts DH, Greenblatt RM, Levine AM, et al. Effects of HIV infection and its treatment on self-reported menstrual abnormalities in women. *J Womens Health (Larchmt)*. 2006;15(5):591–8.
70. Brunham RC, Cheang M, McMaster J, Garnett G, Anderson R. Chlamydia trachomatis, infertility, and population growth in sub-Saharan Africa. *Sex Transm Dis*. 1993;20(3):168–73.
71. Brunham RC, Garnett GP, Swinton J, Anderson RM. Gonococcal infection and human fertility in sub-Saharan Africa. *Proc Biol Sci*. 1991;246(1316):173–7.
72. Coll O, Lopez M, Hernandez S. Fertility choices and management for HIV-positive women. *Curr Opin HIV AIDS*. 2008;3(2):186–92.
73. Bertrand E. Presence of HIV-1 in follicular fluids, flushes and cumulus oophorus cells of HIV-1-seropositive women during assisted reproduction technology. *AIDS*. 2004;18:823–5.
74. Coll O, Fiore S, Floridia M, et al. Pregnancy and HIV infection: A European consensus on management. *AIDS*. 2002;16(suppl 2):S1–18.
75. Martinet V, Manigart Y, Rozenberg S, Becker B, Gerard M, Delvigne A. Ovarian response to stimulation of HIV-positive patients during IVF treatment: A matched, controlled study. *Hum Reprod*. 2006;21(5):1212–7.

76. Barnes A, Riche D, Mena L, Sison T, Barry L, Reddy R, et al. Efficacy and safety of intrauterine insemination and assisted reproductive technology in populations serodiscordant for human immunodeficiency virus: A systematic review and meta-analysis. *Fertil Steril.* 2014;102(2):424–34.
77. Marques C, Guerreiro C, Soares SR. Lights and shadows about the effectiveness of IVF in HIV infected women: A systematic review. *Infect Dis Obstet Gynecol.* 2015;2015:517208.
78. Stora C, Epelboin S, Devouche E, Matheron S, Epelboin L, Yazbeck C, et al. Women infected with human immunodeficiency virus type 1 have poorer assisted reproduction outcomes: A case-control study. *Fertil Steril.* 2016;105(5):1193–201.
79. Coll O, Suy A, Figueiras F, Verneve V, Martinez E, Mataro D, et al. Decreased pregnancy rate after in-vitro fertilization in HIV-infected women receiving HAART. *AIDS.* 2006;20(1):121–3.
80. de Ruiter A, Mercey D, Anderson J, Chakraborty R, Clayden P, Foster G, et al. British HIV Association and Children's HIV Association guidelines for the management of HIV infection in pregnant women 2008. *HIV Med.* 2008;9(7):452–502.
81. Connor EM, Sperling RS, Gelber R, Kiselev P, Scott G, O'Sullivan MJ, et al. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS clinical trials group protocol 076 study group. *N Engl J Med.* 1994;331(18):1173–80.
82. de Ruiter A, Taylor GP, Clayden P, Dhar J, Gandhi K, Gilleece Y, et al. British HIV association guidelines for the management of HIV infection in pregnant women 2012 (2014 interim review). *HIV Med.* 2014;15 Suppl 4:1–77.
83. Gilling-Smith C, Smith JR, Semprini AE, Englert Y, Van Vooren JP, Place I, et al. HIV and infertility: Time to treat. There's no justification for denying treatment to parents who are HIV positive ART in HIV-infected couples: Has the Time come for a change of attitude? *BMJ.* 2001;322(7286):566–7.
84. Ethics Committee of the American Society for Reproductive Medicine. Human immunodeficiency virus and infertility treatment. *Fertil Steril.* 2002;77(2):218–22.
85. Minkoff H, Santoro N. Ethical considerations in the treatment of infertility in women with human immunodeficiency virus infection. *N Engl J Med.* 2000;342(23):1748–50.
86. Lyerly AD, Anderson J. Human immunodeficiency virus and assisted reproduction: Reconsidering evidence, reframing ethics. *Fertil Steril.* 2001;75(5):843–58.
87. Ethics Committee of the American Society for Reproductive Medicine. Human immunodeficiency virus and infertility treatment. *Fertil Steril.* 2010;94(1):11–5.
88. Sauer MV. Providing fertility care to those with HIV: Time to re-examine healthcare policy. *Am J Bioeth.* 2003;3(1):33–40.
89. Gilling-Smith C. Risking parenthood? Serious viral illness, parenting and welfare of the child. In: Shenfield F and Sureau C, (eds.). *Contemporary Ethical Dilemmas in Assisted Reproduction.* 2006. pp. 57–69.
90. David MC, Ha SH, Paynter S, Lau C. A systematic review and meta-analysis of management options for adults who respond poorly to hepatitis B vaccination. *Vaccine.* 2015;33(48):6564–9.
91. Shukla NB, Poles MA. Hepatitis B virus infection: Co-infection with hepatitis C virus, hepatitis D virus, and human immunodeficiency virus. *Clin Liver Dis.* 2004;8(2):445–60.
92. Mast EE, Margolis HS, Fiore AE, Brink EW, Goldstein ST, Wang SA, et al. A comprehensive immunization strategy to eliminate transmission of hepatitis B virus infection in the United States: Recommendations of the advisory committee on immunization practices (ACIP) part 1: immunization of infants, children, and adolescents. *MMWR Recomm Rep.* 2005;54(RR-16):1–31.
93. Schillie S, Murphy TV, Fenlon N, Ko S, Ward JW. Update: Shortened interval for postvaccination serologic testing of infants born to hepatitis B-infected mothers. *MMWR Morb Mortal Wkly Rep.* 2015;64(39):1118–20.
94. Gilling-Smith C, Almeida P. HIV, hepatitis B and hepatitis C and infertility: reducing risk. *Hum Fertil (Camb).* 2003;6(3):106–12.
95. Terrault NA, Dodge JL, Murphy EL, Tavis JE, Kiss A, Levin TR, et al. Sexual transmission of hepatitis C virus among monogamous heterosexual couples: The HCV partners study. *Hepatology.* 2013;57(3):881–9.
96. Chan DP, Sun HY, Wong HT, Lee SS, Hung CC. Sexually acquired hepatitis C virus infection: A review. *Int J Infect Dis.* 2016;49:47–58.
97. Pasquier C, Daudin M, Righi L, Berges L, Thauvin L, Berrebi A, et al. Sperm washing and virus nucleic acid detection to reduce HIV and hepatitis C virus transmission in serodiscordant couples wishing to have children. *AIDS.* 2000;14(14):2093–9.
98. Halfon P, Giorgetti C, Bourliere M, Chabert-Orsoni V, Khiri H, Penaranda G, et al. Medically assisted procreation and transmission of hepatitis C virus: Absence of HCV RNA in purified sperm fraction in HIV co-infected patients. *AIDS.* 2006;20(2):241–6.
99. Bourlet T, Lornage J, Maertens A, Garret AS, Saoudin H, Tardy JC, et al. Prospective evaluation of the threat related to the use of seminal fractions from hepatitis C virus-infected men in assisted reproductive techniques. *Hum Reprod.* 2009;24(3):530–5.
100. Benova L, Mohamoud YA, Calvert C, Abu-Raddad LJ. Vertical transmission of hepatitis C virus: Systematic review and meta-analysis. *Clin Infect Dis.* 2014;59(6):765–73.
101. Checa Cabot CA, Stoszek SK, Quarleri J, Losso MH, Ivalo S, Peixoto MF, et al. Mother-to-child transmission of hepatitis C virus (HCV) among HIV/HCV-coinfected women. *J Pediatric Infect Dis Soc.* 2013;2(2):126–35.
102. Di Domenico C, Di Giacomo C, Marinucci G, Di Paolo A. Vertical transmission of HCV infection: Prospective study in infants born to HIV-1 seronegative women. *Igiene e Sanita Pubblica.* 2006;62(2):129–42.
103. Steyaert SR, Leroux-Roels GG, Dhont M. Infections in IVF: Review and guidelines. *Hum Reprod Update.* 2000;6(5):432–41.
104. Chen H, Ge HS, Lv JQ, Wu XM, Xi HT, Huang JY, et al. Chronic hepatitis B virus infection in women is not associated with IVF/ICSI outcomes. *Arch Gynecol Obstetr.* 2014;289(1):213–7.
105. Lam PM, Suen SH, Lao TT, Cheung LP, Leung TY, Haines C. Hepatitis B infection and outcomes of in vitro fertilization and embryo transfer treatment. *Fertil Steril.* 2010;93(2):480–5.
106. Lorusso F, Palmisano M, Chironna M, Vacca M, Masciandaro P, Bassi E, et al. Impact of chronic viral diseases on semen parameters. *Andrologia.* 2010;42(2):121–6.
107. Englert Y, Moens E, Vannin AS, Liesnard C, Emiliani S, Delbaere A, et al. Impaired ovarian stimulation during in vitro fertilization in women who are seropositive for hepatitis C virus and seronegative for human immunodeficiency virus. *Fertil Steril.* 2007;88(3):607–11.
108. Shaw-Jackson C, Capraro M, Ameye L, Vandromme J, Manigart Y, Rozenberg S, et al. In vitro fertilization for women infected by hepatitis C virus: A matched case-control study and a systematic literature review. *J Assist Reprod Genet.* 2017;34(5):587–97.
109. Kajiyama W, Kashiwagi S, Ikematsu H, Hayashi J, Nomura H, Okochi K. Intrafamilial transmission of adult T cell leukemia virus. *J Infect Dis.* 1986;154(5):851–7.
110. Malm K, Ekeroth B, Hillgren K, Britton S, Fredlund H, Andersson S. Prevalence of human T-lymphotropic virus type 1 and 2 infection in Sweden. *Scandinavian J Infect Dis.* 2012;44(11):852–9.
111. Gessain A, Cassar O. Epidemiological aspects and world distribution of HTLV-1 infection. *Front Microbiol.* 2012;3:388.
112. Recommendations for counseling persons infected with human T-lymphotrophic virus, types I and II. Centers for disease control and prevention and U.S. Public Health Service Working Group. *MMWR Recomm Rep.* 1993;42(RR-9):1–13.
113. Boostani R, Sadeghi R, Sabouri A, Ghabeli-Juibary A. Human T-lymphotropic virus type I and breastfeeding: systematic review and meta-analysis of the literature. *Iran J Neurol.* 2018;17(4):174–9.
114. Hisada M, Maloney EM, Sawada T, Miley WJ, Palmer P, Hanchard B, et al. Virus markers associated with vertical transmission of human T lymphotropic virus type 1 in Jamaica. *Clin Infect Dis.* 2002;34(12):1551–7.

115. D'Ortenzio E, Matheron S, Yazdanpanah Y, de Lamballerie X, Hubert B, Piorkowski G, et al. Evidence of sexual transmission of Zika virus. *N Engl J Med.* 2016;374(22):2195–8.
116. Borges ED, Vireque AA, Berteli TS, Ferreira CR, Silva AS, Navarro PA. An update on the aspects of Zika virus infection on male reproductive system. *J Assist Reprod Genet.* 2019;36(7):1339–49.
117. Petersen LR, Jamieson DJ, Powers AM, Honein MA. Zika virus. *N Engl J Med.* 2016;374(16):1552–63.
118. Mansuy JM, Dutertre M, Mengelle C, Fourcade C, Marchou B, Delobel P, et al. Zika virus: High infectious viral load in semen, a new sexually transmitted pathogen? *The Lancet Infect Dis.* 2016;16(4):405.
119. Reynolds MR, Jones AM, Petersen EE, Lee EH, Rice ME, Bingham A, et al. Vital signs: Update on Zika virus-associated birth defects and evaluation of all U.S. infants with congenital Zika virus exposure - U.S. Zika pregnancy registry, 2016. *MMWR Morb Mortal Wkly Rep.* 2017;66(13):366–73.
120. Barzon L, Pacenti M, Franchin E, Lavezzi E, Trevisan M, Sgarabotto D, et al. Infection dynamics in a traveller with persistent shedding of Zika virus RNA in semen for six months after returning from Haiti to Italy, January 2016. *Euro Surveill.* 2016;21(32):30316.
121. Cao-Lormeau VM, Blake A, Mons S, Lastere S, Roche C, Vanhomwegen J, et al. Guillain-Barre syndrome outbreak associated with Zika virus infection in French Polynesia: A case-control study. *Lancet.* 2016;387(10027):1531–9.
122. Turmel JM, Abgueguen P, Hubert B, Vandamme YM, Maquart M, Le Guillou-Guillemette H, et al. Late sexual transmission of Zika virus related to persistence in the semen. *Lancet.* 2016;387(10037):2501.
123. Gaskell KM, Houlihan C, Nastouli E, Checkley AM. Persistent Zika virus detection in semen in a traveler returning to the United Kingdom from Brazil, 2016. *Emerg Infect Dis.* 2017;23(1):137–9.
124. Filho EA, Facio CL, Machado-Paula LA, Oliveira MA, Martinhago CD, Araujo LP, et al. Case report of Zika virus during controlled ovarian hyperstimulation: Results from follicular fluid, cumulus cells and oocytes. *JBRA Assist Reprod.* 2019;23(2):172–4.
125. Joguet G, Mansuy JM, Matusali G, Hamdi S, Walschaerts M, Pavili L, et al. Effect of acute Zika virus infection on sperm and virus clearance in body fluids: A prospective observational study. *Lancet Infect Dis.* 2017;17(11):1200–8.
126. Khalil A, Kalafat E, Benlioglu C, O'Brien P, Morris E, Draycott T, et al. SARS-CoV-2 infection in pregnancy: A systematic review and meta-analysis of clinical features and pregnancy outcomes. *EClinicalMedicine.* 2020;25:100446.
127. Knight M, Bunch K, Vousden N, Morris E, Simpson N, Gale C, et al. Characteristics and outcomes of pregnant women admitted to hospital with confirmed SARS-CoV-2 infection in UK: National population based cohort study. *BMJ.* 2020;369:m2107.
128. Balachandren N, Barrett G, Stephenson JM, Yasmin E, Mavrelis D, Davies M, et al. Impact of the SARS-CoV-2 pandemic on access to contraception and pregnancy intentions: A national prospective cohort study of the UK population. *BMJ Sex Reprod Health.* 2022;48(1):60–5.
129. Collins AB, Zhao L, Zhu Z, Givens NT, Bai Q, Wakefield MR, et al. Impact of COVID-19 on male fertility. *Urology.* 2022;164:33–9.
130. Gonzalez DC, Nassau DE, Khodamoradi K, Ibrahim E, Blachman-Braun R, Ory J, et al. Sperm parameters before and after COVID-19 mRNA vaccination. *JAMA.* 2021;326(3):273–4.
131. Mohr-Sasson A, Haas J, Abuhasira S, Sivan M, Doitch Amdurski H, Dadon T, et al. The effect of covid-19 mRNA vaccine on serum anti-Mullerian hormone levels. *Hum Reprod.* 2022;37(3):534–41.
132. Orvieto R, Segev-Zahav A, Aizer A. Does COVID-19 infection influence patients' performance during IVF-ET cycle? An observational study. *Gynecol Endocrinol.* 2021;37(10):895–7.
133. Orvieto R, Noach-Hirsh M, Segev-Zahav A, Haas J, Nahum R, Aizer A. Does mRNA SARS-CoV-2 vaccine influence patients' performance during IVF-ET cycle? *Reprod Biol Endocrinol.* 2021;19(1):69.
134. Sharp GC, Fraser A, Sawyer G, Kountourides G, Easey KE, Ford G, et al. The COVID-19 pandemic and the menstrual cycle: Research gaps and opportunities. *Int J Epidemiol.* 2022;51(3):691–700.
135. Zauche LH, Wallace B, Smoots AN, Olson CK, Oduyebo T, Kim SY, et al. Receipt of mRNA covid-19 vaccines and risk of spontaneous abortion. *N Engl J Med.* 2021;385(16):1533–5.
136. Wang M, Yang Q, Ren X, Hu J, Li Z, Long R, et al. Investigating the impact of asymptomatic or mild SARS-CoV-2 infection on female fertility and in vitro fertilization outcomes: A retrospective cohort study. *EClinicalMedicine.* 2021;38:101013.
137. Gilling-Smith C, Emiliani S, Almeida P, Liesnard C, Englert Y. Laboratory safety during assisted reproduction in patients with blood-borne viruses. *Hum Reprod.* 2005;20(6):1433–8.
138. Lesourd F, Izopet J, Mervan C, Payen JL, Sandres K, Monrozies X, et al. Transmissions of hepatitis C virus during the ancillary procedures for assisted conception. *Hum Reprod.* 2000;15(5):1083–5.
139. Cobo A, Bellver J, de Los Santos MJ, Remohi J. Viral screening of spent culture media and liquid nitrogen samples of oocytes and embryos from hepatitis B, hepatitis C, and human immunodeficiency virus chronically infected women undergoing in vitro fertilization cycles. *Fertil Steril.* 2011;97(1):74–8.
140. Benifla JL, Letur-Konirsch H, Collin G, Devaux A, Kuttenn F, Madelenat P, et al. Safety of cryopreservation straws for human gametes or embryos: A preliminary study with human immunodeficiency virus-1. *Hum Reprod.* 2000;15(10):2186–9.
141. Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AE, Fielding A, Briggs EM, et al. Hepatitis B transmission from contaminated cryopreservation tank. *Lancet.* 1995;346(8968):137–40.
142. Clarke GN. Sperm cryopreservation: Is there a significant risk of cross-contamination? *Hum Reprod.* 1999;14(12):2941–3.
143. Tomlinson M, Sakkas D. Is a review of standard procedures for cryopreservation needed?: Safe and effective cryopreservation—should sperm banks and fertility centres move toward storage in nitrogen vapour? *Hum Reprod.* 2000;15(12):2460–3.

FERTILITY OPTIONS FOR TRANSGENDER AND NONBINARY INDIVIDUALS

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Introduction

As of 2016, there were an estimated 1.4 million transgender individuals living in the United States, representing 0.6% of adults [1]. A 2020 systematic review estimates that 0.3%–0.5% of adults and 1.2% to 2.7% of children and adolescents identify as transgender worldwide. When expanded to include nonbinary and other gender-diverse identities these estimates increased to 0.5%–4.5% of adults and 2.5%–8.4% of children and adolescents [2]. Extrapolating from an estimate of 0.5% of the adult population, there are approximately 25 million individuals that identify as transgender worldwide [3]. While obtaining accurate estimates of transgender and nonbinary (TGNB) individuals can be challenging, secondary to a lack of inclusion or consistency of gender identity on survey studies as well as hesitancy to report such identity due to societal stigmatization, epidemiologic data indicate that TGNB individuals represent a sizeable and increasing proportion of the general population. This community has faced a longstanding history of prejudice and discrimination and remain an underserved population in healthcare. Healthy People 2030 identified this need for improved care for gender diverse individuals mandating gender identity data collection in all Health and Human Services efforts and investing in programmes to address stigmatization of TGNB youth [4]. Unfortunately, reproductive health and fertility care is no exception, as there are significant barriers to access and a lack of high-quality care standards for TGNB individuals.

An understanding of gender identity is integral to the discussion and provision of fertility care for TGNB individuals (Table 68.1). Gender identity is best viewed as a spectrum rather than binary entity and refers to one's internal experience of gender or genders. This does not have to be a fixed identity and gender fluid refers to one's view of their gender as evolving or fluctuating. Importantly, one's gender identity is distinct from and does not define their sexual orientation, which refers to one's attraction to individuals of a specific or more than one gender. Nonbinary refers to the gender identity of an individual who does not identify according to a binary sense of gender. A transgender individual is one whose gender identity differs from their sex assigned at birth. A cisgender individual is one whose sex assigned at birth corresponds to their gender identity. Gender dysphoria refers to distress caused by a discordance between one's sex assigned at birth and gender identity. In order to meet criteria according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) this distress must be clinically significant and cause an impairment in functioning [5]. The degree to which individuals experience gender dysphoria is highly variable and may impact one's decision to pursue a gender transition. An individual may elect to pursue a gender transition by modifying their physical appearance to reflect their gender identity and this may include gender-affirming hormonal treatment and/or gender-affirming surgical

treatment. The decision to pursue a gender transition and inclusion of hormonal or surgical treatments is uniquely personal and may be influenced by one's ability to access gender-affirming care.

TGNB parenting

According to national survey studies, approximately a quarter to a half of transgender individuals are current parents, with the majority becoming parents prior to gender transition [6–8]. A recent national probability sample of TGNB adults in the United States found that only 18.8% of respondents were parents, with transgender women (52.5%) and nonbinary (35.8%) respondents significantly more likely to be parents as compared to transgender men (11.7%) [9]. Despite these relatively low rates of parenting among TGNB adults, survey studies of family planning found significant interest in future parenting among this community [8, 10–12]. A large multicentre study in Germany found that 69.9% of transgender women and 46.9% of transgender men were interested in future children [10]. This discrepancy between desire for parenthood and achieving parenthood likely reflects stigmatization and significant limitations in access to reproductive health, fertility, and family-building resources. Interest in future parenting was similarly high among transgender young adults with one-third to two-thirds of respondents reporting a desire to parent in their lifetime [13]. Among transgender youth with desire for future parenthood there was less emphasis on having biologically related children, with 31.3% reporting that they would plan to build their families via adoption [14].

TGNB individuals desire to parent for the same reasons as cisgender individuals including family-building, nurturance and closeness [15]. Historically, discrimination on the basis of gender identity has influenced perceptions of TGNB people's fitness to parent. In describing concern regarding the use of ART among transgender people, one reproductive endocrinologist wrote: "Most patients with transsexuality seem to have additional types of aberrant behavior ... the rejection rate should be very high" [16]. Conversely, limited studies of transgender individuals as parents show the same qualities of warmth, commitment and attention to a child's needs as cisgender individuals [7]. Stigmatization of TGNB people have likewise propelled myths that children born to TGNB individuals are at risk for abnormal childhood development. However, multiple recent studies of childhood development have dispelled this myth, showing no impact of transgender parenting on developmental milestones [17–19]. One recent prospective cohort study of 32 children conceived by donor sperm insemination among French couples with a cisgender woman and a transgender man showed no evidence to suggest that having a transgender parent affects a child's gender identity, mental health or cognitive development [19]. In an effort to dispel this unfounded perception, the American Academy of Child and Adolescent Psychiatry has stated "there is no credible evidence

TABLE 68.1 Glossary of Terms Relating to Gender Identity and Expression

Gender identity	An individual's internal experience of gender
Sexual identity	How an individual characterizes their emotional or sexual attraction towards one or more genders
Gender expression	The outward manner in which an individual displays their gender
Cisgender	Describes an individual whose gender identity corresponds to their sex assigned at birth
Transgender	Describes an individual whose gender identity differs from their sex assigned at birth
Trans man / Transgender man	A transgender individual whose gender identity is male
Trans woman / Transgender woman	A transgender individual whose gender identity is female
Nonbinary	Describes an individual whose gender identity does not exclusively fit categories of girl/woman or boy/man but rather who experiences gender outside of this binary definition
Gender diverse	Describes a community of people who fall outside of a binary structure of gender
Gender fluid	Describes an individual whose gender identity is not fixed
Gender dysphoria	Distress that an individual experiences secondary to discordance between one's gender identity and sex assigned at birth. To meet criteria, this distress must be clinically significant and cause an impairment in functioning.
Gender-affirming hormone therapy	Feminizing and masculinizing hormone treatment aimed at aligning secondary sexual characteristics with gender identity
Gender-affirming surgery	Surgeries aimed to modify a person's body to have greater alignment with their gender identity
Misgender	To refer to an individual by a pronoun or other gendered term (i.e. Ms/Mr) that incorrectly indicates their gender identity

Note: Definitions adopted from Fenway Institute National LGBTQIA+ Health Education Center Glossary of Terms for Healthcare Teams, 2020, and UCSF Transgender Care Terminology and Definitions, 2016.

that shows that a parent's sexual orientation or gender identity will adversely affect the development of the child" [20]. Despite the scientific literature, there are recent reports of transgender individuals being denied fertility services on the basis of their gender identity [12, 21, 22]. In an effort to combat this continued discrimination in the fertility sphere, the American Society of Reproductive Medicine (ASRM) issued a 2021 Ethics Committee Opinion calling for programmes to treat all requests for assisted reproduction without regard to gender identity and stating that "professional autonomy, although a significant value in deciding whom to treat, is limited in this case by a greater ethical obligation, and in some jurisdictions a legal duty, to regards all persons equally regardless of their gender identity" [15].

Barriers to care

The discrepancy between the proportion of TGNB individuals with desire to parent and the proportion that are able to parent is largely reflective of the significant stigmatization and barriers to adequate healthcare that this population faces. Prejudice and discrimination on the basis of gender identity has influenced the education, economic, healthcare and legal systems such that TGNB individuals face a disproportionate challenge to have their basic health needs met. According to the US 2015 Transgender Survey, 29% of transgender respondents were currently living in poverty, 15% were unemployed (three times the national rate) and 30% had experienced homelessness in their lifetime with 12% experiencing homelessness in the past year [23]. In the education system, more than half of transgender individuals reported being verbally harassed, 24% physically attacked, and 17% report leaving school secondary due to severe mistreatment. Forty-six per cent of respondents reported harassment on the basis of their gender identity in the past year, and a striking 40% had attempted suicide in their lifetime, nearly nine times the national rate [23]. This discrimination of TGNB people is not unique to the United States, and in a survey of more than 500 transgender individuals in Belgium, 38% of respondents reported fear of their child being discriminated against due to having a transgender parent, and a third of respondents noted fear of discrimination as a transgender parent as barriers to achieving parenthood [11].

Historic discrimination of TGNB individuals and fear of TGNB procreation is deeply rooted in the legal system. In 1972, Sweden was the first European country to formally acknowledge the preferred identity of transgender individuals. However, applicants for legal gender recognition were required to prove an incapacity to reproduce [24]. Many other countries worldwide passed laws in this manner similarly requiring sterilization as a prerequisite for legal gender change [25]. It wasn't until the most recent decade that these transgender sterilization laws have faced significant scrutiny, with the landmark 2017 opinion of the European Court of Human Rights ruling that "by conditioning gender recognition on submission to a sterilization operation ... France had violated the applicants' right to private life" [26]. Despite this ruling and similar overturning of sterilization requirements for legal gender change in Germany, Sweden, and Italy, as of 2019 there are 16 countries in Europe and Central Asia, including Finland, Czech Republic, and Turkey, that continue to require sterilization of trans persons seeking recognition of their gender identity [27]. Beyond legal requirements for recognition of preferred gender, TGNB individuals have faced additional legal barriers including cases of gender identity being used as a basis for denying or restricting custody or visitation of gender minorities [28–33]. In one survey of more than 6000 transgender adults, among respondents who had children and were in a relationship that ended, 13% reported that a court stopped or limited their relationships with children because of their transgender identity [6].

Unfortunately, this discrimination and prejudice on the basis of one's gender identity also permeates the healthcare system. In the United States, a third of transgender individuals reported at least one negative experience in healthcare secondary to their gender identity and 23% reported that they did not see a doctor when they knew they needed to secondary to fear of mistreatment [23]. Similarly, 22% of transgender respondents on European Union Agency for Fundamental Rights Survey reported being personally

discriminated against by healthcare personnel because of their gender identity [34]. In another survey of transgender adults, 71% of respondents reported at least one instance of mistreatment in healthcare, with 23% describing more than one instance. This mistreatment in healthcare of transgender individuals included gender insensitivity, displays of discomfort, denial of services, substandard care, verbal abuse, and forced care [35]. This perception by TGNB patients of provider discomfort and substandard care is not surprising given the lack of formal medical teaching relating to the unique healthcare needs of these communities. Assessments by medical school deans in the United States and Canada revealed that there was a median of only five hours of LGBT content in the entire curriculum with a third of schools citing zero hours relating to care of LGBT patients [36]. Survey studies of primary care providers as well as endocrinologists similarly reflect a lack of formal training and comfort in treating TGNB individuals [37–40].

A discussion of healthcare barriers to reproductive care for TGNB individuals must also include insurance coverage. Prior to the implementation of the Affordable Care Act (ACA) in 2010, being transgender was considered a “pre-existing” condition with TGNB individuals routinely denied coverage. The ACA prohibits discrimination based on sex and in 2016 was updated to include discrimination based on gender identity [41]. However, insurance coverage for gender-affirming care remains inconsistent, with over half of transgender individuals reporting being denied coverage for gender-affirming surgery and one-quarter denied coverage for gender-affirming hormones [23]. Additionally, survey studies of TGNB individuals interested in parenting or fertility preservation consistently cite cost of treatment/lack of insurance coverage as a primary obstacle to pursuing treatment [11, 42]. In addition to gaps in coverage, limitations of electronic health records that presume a binary definition of gender and/or link a patient’s registered sex with presumed organs can lead to routine denial of coverage for appropriate screening and treatments [43]. Insurance requirements or negotiation of coverage may also “out” patients representing further barriers to coverage for TGNB individuals.

In addition to the aforementioned barriers, there are aspects inherent to fertility preservation and fertility treatments that can invoke gender dysphoria for TGNB individuals. For some TGNB individuals considering fertility treatments and discussion of gonads and reproductive potential can be challenging. For individuals with ovaries, fertility testing and monitoring often involves pelvic ultrasounds with oocyte retrievals traditionally performed via a transvaginal approach. While transabdominal ultrasounds and oocyte retrievals can be pursued to limit gender dysphoria, the success of such approach is dependent upon an individual’s body habitus and anatomy. Potential discontinuation of androgen treatment prior to or during oocyte stimulation as well as side effects from gonadotropin medications can cause significant gender dysphoria, and fear of these changes prohibits some individuals from pursuing treatment [44, 45]. For transfeminine individuals pursuing feminizing hormone treatment, fertility preservation and treatment may require discontinuation of hormone therapy and involve masturbation to produce a semen sample, both of which can be very dysphoric [46, 47]. Some transfeminine people, particularly those with prior hormone treatment, may be psychologically or physically unable to produce a sample and alternative options for sperm collection should be discussed. Many TGNB individuals initiate gender-affirming care at a young age with their youth and emphasis on pursuing transition

often preventing consideration of future fertility needs. In a survey of transgender adolescents, 31% did not feel that they could make a meaningful decision about fertility at that time and only 3% were willing to delay gender-affirming hormones for fertility preservation [48]. Unfortunately, this can lead to significant decisional regret and highlights the importance of thorough fertility preservation counselling with inclusion of strategies to minimize delay of initiation of gender-affirming treatments.

Gender-affirming treatments

TGNB individuals may elect to take steps to better align their outward physical appearance with their gender identity in an effort to decrease gender dysphoria. The decision to transition is highly personalized and may include a social transition without treatment, medical transition with hormone treatment, and/or undergoing minor or major gender-affirming surgical procedures. The impact of gender-affirming surgical treatments on reproductive potential is procedure-specific. Transmasculine individuals who desire a more masculine appearance may pursue chest masculinization (top surgery), which does not impact one’s reproductive potential but may affect one’s future ability to breastfeed [49]. In contrast, other major gender-affirming surgical treatments directly impact one’s reproductive potential, with a hysterectomy eliminating one’s ability to carry a pregnancy and bilateral salpingo-oophorectomy eliminating one’s ability to provide future reproductive gametes (unless gametes, embryos, or reproductive tissues were previously cryopreserved or cryopreserved at time of surgery). For transfeminine individuals pursuing feminizing gender-affirming surgeries, future reproductive potential is dependent upon whether an orchectomy is pursued (again, unless previous cryopreservation of sperm or testicular tissue has occurred). Given the definitive impact of many major gender-affirming surgical treatments on an individual’s reproductive potential, it is critical that TGNB individuals receive thorough fertility preservation counselling prior to these procedures.

Transmasculine individuals who desire a more masculine appearance may pursue gender-affirming hormone treatment with testosterone. Testosterone is most often administered as a weekly or biweekly injection (either intramuscular or subcutaneous) or via transdermal application (either patch or gel). The goal of testosterone as gender-affirming hormone treatment is to evoke secondary sex characteristics associated with one’s gender identity while minimizing secondary sex characteristics of one’s natal sex. This is achieved through suppression of oestradiol with the goal of increasing testosterone to physiologic male reference levels. Anticipated physical changes may include deepening of the voice, fat redistribution, increased muscle mass, clitoromegaly, cessation of menses, increased facial hair growth, and decreased breast size.

The mainstay of feminizing hormone treatment is oestradiol in combination with an anti-androgen. Oestradiol can be administered by a variety of routes including oral, sublingual, transdermal, and via intramuscular injection. The most common anti-androgen for gender-affirming hormone treatment in the United States is spironolactone, which is an oral tablet typically dosed twice daily. In a manner similar to masculinizing hormone treatment, the goal of oestradiol in combination with an anti-androgen is to minimize natal secondary sex characteristics and induce secondary sex characteristics consistent with one’s gender identity. Serum hormone levels are regularly monitored with the goal of achieving physiologic female reference range oestradiol

and testosterone levels. Physical changes anticipated with feminizing hormone treatment include breast development, reduction of terminal body hair, and fat redistribution.

Testosterone and reproductive potential

Testosterone as gender-affirming hormone treatment induces amenorrhea in the majority of patients, likely through a combination of ovulation suppression and induction of endometrial inactivity or atrophy. In studies of transgender men on testosterone, the mean time to achieving amenorrhea was three months, with more than 90% achieving amenorrhea by six months of treatment [50–53]. Testosterone-induced amenorrhea is a dose-dependent effect with higher rates of amenorrhea achieved with physiologic male range hormone levels [51]. In a recent prospective cohort of 267 transgender men initiating testosterone treatment, 82% were amenorrhoeic after three months and 91% after six months of testosterone administration [54]. It is important to acknowledge and communicate to patients that while the majority of individuals will experience amenorrhea with testosterone treatment the ovulation suppression is likely incomplete as evidenced by case reports of pregnancies in the setting of testosterone-induced amenorrhea [55, 56]. At the level of the endometrium, histologic studies show variable effects of testosterone. In some studies, long-term testosterone administration induced endometrial inactivity or atrophy in the majority of specimen [57, 58] with others showed greater heterogeneity with the presence of active endometrium in many specimen [59, 60]. An analysis of endometrial samples after one year of testosterone treatment showed similar levels of Ki-67, a marker of endometrial proliferation, as samples from postmenopausal women favouring an atrophic effect of long-term testosterone treatment [61].

At the level of the ovary, testosterone treatment has shown mixed results on ovarian structure and folliculogenesis. In a mouse model of testosterone as gender-affirming hormone treatment, testosterone treated mice had an absence of corpus lutei without a reduction in primordial, primary, secondary, or total antral follicle counts [62]. These results are similar to other recent studies of ovaries from transgender men in which testosterone treatment did not alter the number or distribution of follicles [63, 64]. A recent analysis of ovaries at the time of gender-affirming surgery from 85 transgender men with prior testosterone treatment showed persistent ovarian function in the majority of specimen with follicular/simple cysts in 49%, normal pathology in 38.8%, and polycystic morphology in 5.9%. This benign spectrum of pathology was within the normal range for reproductive-age ovaries and there was no association between duration of testosterone and presence of cysts [65]. In another histopathologic analysis of ovaries from transgender men with prior testosterone treatment, fluorescence activated cell sorting analysis revealed the majority of ovarian cells to be vital (88%) with normal cortical follicle distribution [66]. These results are in contrast to older studies of ovarian pathology in transgender men with previous testosterone treatment in which polycystic ovarian morphology or atrophic findings were predominant [58, 67].

There are limited studies investigating ovarian reserve in the setting of testosterone treatment which show disparate findings. In one study, Caanen and colleagues found a significant reduction in AMH concentrations after 16 weeks of androgen treatment. However, the individuals in the study protocol were also treated with GnRH agonist and an aromatase inhibitor limiting the ability to draw conclusions on the impact of testosterone alone [68].

In another analysis of transgender adolescents receiving testosterone treatment for over six months, AMH levels remained stable throughout treatment. Subjects in this cohort received testosterone in combination with Lynestrenol, an androgenic progestin, which may have influenced these findings [69]. A more recent investigation of ovarian reserve in transgender men found that after one year of testosterone treatment there was no difference in antral follicle count and, after excluding individuals with PCOS, no difference in AMH levels [70]. Although limited by small sample size and duration of testosterone treatment, these findings suggest preserved ovarian reserve following testosterone treatment.

There is limited evidence for the impact of long-term testosterone treatment on oocyte quality with further research needed to elucidate this impact and determine if there is reversibility with testosterone cessation. In a mouse model of short-term testosterone treatment, Bartels and colleagues found no difference in retrieved oocytes following gonadotropin stimulation, *in vitro* fertilization, or 2-cell embryo development [71]. However, another investigation of IVF outcomes following short-term testosterone treatment found impaired fertilization of oocytes from testosterone treated female mice as compared to controls which resolved following a washout period [72]. Early studies of *in vitro* maturation from cumulus–oocyte complexes collected from antral follicles present in the ovaries of transgender men after prior testosterone treatment revealed a high percentage of normal appearing spindles and chromosome alignment, markers of oocyte functionality [73, 74]. However, a more recent study of ovarian tissue oocyte *in vitro* maturation among 83 transgender men with prior testosterone treatment found poor rates of maturation (23.8%), fertilization (34.5%), and day 3 embryo development (52.1%). Morphokinetic analysis revealed aberrant cleavage patterns and early embryo arrest indicating poor developmental capacity of the oocytes derived from ovarian tissue with prior testosterone exposure [75]. While not conclusive, there is current evidence which supports a potential detrimental impact of testosterone on oocyte function and fertility highlighting the importance of education and fertility preservation counselling for the TGNB community prior to initiation of masculinizing hormone treatment.

Feminizing hormones and reproductive potential

Histologic studies of prolonged oestradiol treatment have shown testicular damage and variable suppression of spermatogenesis. Analysis of orchectomy specimen from transgender women with prior feminizing hormone treatment have ranged from 24% normal spermatogenesis to a complete lack of normal spermatogenesis [76–82]. Testicular tissue analysis from a recent prospective cohort of 97 transgender women with a mean duration of feminizing hormone treatment of 685 days prior to gonadectomy revealed the absence of normal spermatogenesis in any specimen. Immunostaining revealed partial spermatogenesis in 22.7%, early maturation arrest in 8.2%, late maturation arrest in 14.4%, and complete absence of germ cells in 12.4% of specimens. Higher serum testosterone levels were associated with more advanced maturation and higher oestradiol levels with lower number of spermatogonia, which suggests a possible dose-dependent relationship between oestradiol and impaired fertility [80]. Another histological study compared orchectomy specimen from transgender women that initiated care in adolescence versus

adulthood and found that testicular histology and spermatogenesis was more negatively affected in those that initiated care as adults. In this study cessation of hormonal treatment prior to gonadectomy was not associated with maturation stage or presence of germ cells, however, duration of cessation was unknown [81]. In an analysis of testicular histology of orchectomy specimen from transgender women with prior feminizing treatment 28.2% of specimen revealed hyalinization, 20% scarring or fibrosis, and 25.9% testicular atrophy [79]. Interestingly the duration of hormone treatment was not associated with degree of spermatogenesis or presence of pathologic changes [78, 79, 82]. Additional pathologic changes identified in orchectomy specimen of transgender women with prior feminizing hormone treatment irrespective of prior anti-androgen use include decreased seminiferous tubule diameter, abnormal appearance of Sertoli or Leydig cells and fatty degeneration of connective tissue [77, 78, 83]. Evidence from orchectomy specimen from transgender women indicate varying degrees of impaired reproductive potential with feminizing hormone therapy, but further studies are needed to ascertain whether these effects are dose or regimen dependent and whether they exhibit reversibility with treatment discontinuation.

Feminizing hormone treatment has been associated with diminished sperm parameters in transgender women, but there is evidence of potential reversibility of this impact on reproductive function with treatment discontinuation. An early investigation of ethinyl oestradiol and semen quality found a dose-dependent impact on sperm concentration and motility which was reversible with discontinuation [84]. A mouse model of increasing oestradiol dosing similarly found a dose-dependent effect on sperm count, motility, morphology and DNA fragmentation [85]. Recent investigations of cryopreserved sperm samples from transgender women have demonstrated reductions in concentration, per cent motility, and total motile sperm counts [86, 87]. Importantly, specimens collected after hormone discontinuation were comparable with transgender women who were hormone naïve indicating potential reversibility of this impact [86]. It is worth noting that in multiple investigations of cryopreserved sperm samples from transgender women, individuals with no prior hormone treatment had reduced sperm parameters as compared to WHO reference standards even after controlling for known risk factors including obesity, alcohol use, and cannabis use [87, 88]. There is conflicting data as to whether behavioural modifications such as wearing tight undergarments and tucking account for this reduction in sperm parameters or whether there are other risk factors or social determinants of health that negatively impact the reproductive potential of transgender women [89–91]. While the cause of this reduction in sperm parameters in hormone-naïve transgender women remains yet to be identified, the low sperm counts of many transgender women prior to initiation of hormone treatment is important to consider in fertility preservation counseling and family planning discussions.

Fertility preservation

There is consensus among major medical societies including World Professional Association for Transgender Health (WPATH), Endocrine Society, ASRM, and European Society of Human Reproduction and Embryology (ESHRE) which call for universal fertility preservation counselling for all TGNB individuals prior to initiation of gender-affirming treatment as standard of care [15, 22, 92, 93]. ASRM goes further to state that fertility preservation counselling for TGNB individuals should include

discussion of the limited data on long-term outcomes for patients and their offspring [15]. Despite these universal recommendations, fertility preservation counselling is inconsistent and often incomplete prior to gender-affirming treatments contributing to a lack of awareness and likely decreased uptake of fertility preservation services [11–13, 94]. However, even among studies with high rates of fertility preservation counselling, use of fertility preservation services remained low particularly among TGNB youth [13, 46, 95]. The low uptake of fertility preservation services among TGNB adolescents reflects the additional challenges of anticipating family planning desires at a young age and unwillingness to delay gender transition. An additional consideration for fertility preservation among TGNB individuals is discussion of potential third party reproduction and FDA testing requirements including a physical exam, review of medical history with assessment of risk factors and STI testing. For individuals who have uncertain partner status or family building goals at the time of fertility preservation, completion of FDA testing at the time of gamete preservation is recommended so that reproductive options are not limited to a sexually intimate partner and a gestational carrier may be considered in the future.

Fertility preservation options for transmasculine people

Fertility preservation options for transmasculine individuals with ovaries include oocytes cryopreservation, embryo cryopreservation, and ovarian tissue cryopreservation. The method to achieve parenthood, advantages, disadvantages, and strategies to reduce gender dysphoria for each of these fertility preservation options are outlined in Table 68.2. As compared with options for transfeminine people, fertility preservation options for transmasculine people can be intensive and result in gender dysphoria, which may contribute to low uptake. Although there are case reports of prepubertal ovarian stimulation, oocyte and embryo cryopreservation are generally restricted to post-pubertal individuals due to concerns regarding the immature hypothalamic–pituitary–ovarian axis [96, 97]. A potential exception is prepubertal transmasculine youth who started puberty blockade at Tanner stage II or III in whom ovarian stimulation may be considered. For TGNB individuals that have not reached puberty, the only current method of fertility preservation is ovarian tissue cryopreservation. All methods of fertility preservation present unique challenges for TGNB individuals with the potential to invoke gender dysphoria, and a qualified mental health professional should be included in the individual's care team to assist with navigating these challenges [15].

Oocyte and embryo cryopreservation

Oocyte and embryo cryopreservation are highly effective methods of fertility preservation with early data in TGNB individuals showing promising results. The decision of whether to preserve oocytes versus embryos is highly personal and is often influenced by an individual's age at time of fertility preservation, partner status and reproductive organs of their partner and whether they desire to use them. As many TGNB individuals desire to initiate gender-affirming treatments at a young age, oocyte cryopreservation allows the opportunity to preserve reproductive gametes and have individual ownership over those gametes without having to make decisions regarding future family building at the time of cryopreservation. On the other hand, for individuals who are older or have found the partner they want to build a family with, embryo cryopreservation offers the ability for partner

TABLE 68.2 Fertility Preservation Options for Transmasculine Individuals with Ovaries

Technique	Means to Achieve Parenthood	Advantages	Disadvantages	Strategies to Reduce Gender Dysphoria
Oocyte Cryopreservation	- oocyte can be fertilized in the future with partner or donor sperm - resultant embryos can be transferred to individual, partner with a uterus or gestational carrier	- highly effective method of fertility preservation - early studies show promising results in transmasculine individuals - does not require partner or donor at time of preservation - individual ownership over reproductive gametes	- cost - intensive two-week process involving ultrasounds, injections and retrieval - oocyte retrieval typically requires IV anaesthesia - process and medications can cause gender dysphoria - lack of data regarding T discontinuation	- consider transabdominal or transrectal ultrasound approach when able - aromatase inhibitor during stimulation to limit oestradiol rise - random start protocol to minimize delay in gender transition - weigh individual risks and benefits of T discontinuation - consultation with qualified mental health professional
Embryo Cryopreservation	- embryos can be transferred to individual, partner with a uterus or gestational carrier - embryos may be transferred fresh or frozen for future use	- highly effective method of fertility preservation - early studies show promising results in transmasculine individuals - allows for genetic testing of embryo for aneuploidy and/or inherited disorder - if partner has sperm allows for both partners to preserve gametes together	- includes disadvantages of oocyte cryopreservation - requires partner with sperm or donor selection at time of preservation - if using partner sperm may no longer have individual ownership over reproductive material	- same considerations as oocyte cryopreservation
Ovarian Tissue Cryopreservation	- autologous transplant of cryopreserved ovarian tissue to achieve future pregnancy - following autologous transplant may undergo future stimulation and IVF with option for embryo transfer to individual, partner with a uterus or gestational carrier	- only fertility preservation option available for prepubertal individuals (also available for post-pubertal individuals) - potential future option for <i>in vitro</i> maturation of oocytes from cryopreserved ovarian tissue (experimental) - avoids present need for ovarian stimulation - individual ownership over reproductive tissue	- requires abdominal surgery (often laparoscopic) - future autologous transplant may be gender dysphoric - future ovarian stimulation may be gender dysphoric - limited data on long-term and offspring outcomes	- ovarian tissue may be retrieved at the time of gender-affirming surgery (limiting or eliminating delay to gender transition) - all pregnancies to date have been in the setting of autologous transplant - consultation with qualified mental health professional

participation. If a partner has the capacity to produce sperm it may be used for fertilization or if the partner has a uterus and is willing to carry a pregnancy a resultant embryo may be transferred to the partner via reciprocal IVF. If neither the patient nor a partner have a uterus in which they are willing to carry a pregnancy, FDA testing (outlined above) should be performed at the time of cryopreservation to better facilitate use of a gestational carrier in the future. Creation of embryos also allows for the option of genetic testing (PGT-A and/or PGT-M) and improved counselling regarding probability of achieving a live birth from the reproductive tissues obtained.

Ovarian tissue cryopreservation

Ovarian tissue cryopreservation is a fertility preservation procedure in which ovarian tissue (both ovaries, one ovary, or segments of ovarian tissue) is surgically removed, typically via a laparoscopic approach, and small pieces of ovarian tissue cortex are cryopreserved for future use. Until recently, ovarian tissue

cryopreservation was considered an experimental technique performed under research protocols at a finite number of institutions. However, in 2019 ASRM lifted its experimental status, and an increasing number of institutions are now offering this procedure [98]. As of 2017, there were more than 130 live births reported following autologous transplantation of previously cryopreserved ovarian tissue worldwide, half of which were naturally conceived [99]. There are many aspects of ovarian tissue cryopreservation that may be advantageous to the TGNB community including the ability to preserve ovarian tissue at the time of gender-affirming surgery and the potential to avoid the process of ovarian stimulation, which can invoke gender dysphoria and delay gender transition. However, all live births resulting from ovarian tissue cryopreservation to date have occurred in the setting of autologous transplantation, which may be highly unfavourable to individuals that have already pursued a gender transition or who are not interested in carrying a pregnancy. *In vitro* maturation of oocytes obtained from ovarian tissue cryopreservation offers a

potential future option of utilizing this cryopreserved tissue that would significantly expand reproductive options for the TGNB community.

Fertility preservation options for transfeminine people

For transfeminine individuals, fertility preservation options include sperm cryopreservation, embryo cryopreservation, and testicular tissue cryopreservation (Table 68.3). Testicular tissue cryopreservation is currently the only fertility preservation option for prepubertal individuals assigned male at birth and continued scientific advances are needed to allow cryopreserved immature testicular tissue to be used for human reproduction. In contrast to fertility options for transmasculine people, fertility preservation for transfeminine people via sperm cryopreservation is significantly less laborious and costly. As such, studies of fertility preservation among transgender individuals often show similar interest in preserving fertility between transgender men and transgender women with higher uptake of fertility preservation services (sperm cryopreservation) among transgender women as compared to transgender men [10, 13].

Sperm cryopreservation

Sperm cryopreservation is a relatively low cost and highly effective method of fertility preservation for transfeminine individuals with testes. For individuals with sperm parameters within normal range, sperm cryopreservation allows for fertility preservation with the option to use the sperm for intrauterine insemination or *in vitro* fertilization in the future. However, TGNB individuals can face unique challenges in the setting of sperm cryopreservation. Sperm is traditionally collected via production of a masturbatory sample, but for some transfeminine individuals this may not be possible secondary to the effects of prior feminizing hormone treatment or significant gender dysphoria [46, 47]. Fertility preservation counselling for transfeminine people should include discussion of alternate methods of sperm extraction such as electroejaculation, aspiration, or surgical extraction for individuals who are unable to produce an ejaculate or who have diminished sperm parameters, such as oligospermia or obstructive azoospermia, which necessitate a urologic procedure. An additional consideration in the setting of sperm cryopreservation unique to this population is the evidence from multiple studies of diminished semen parameters in hormone naïve transgender women [87–91]. In a recent cohort study of more than 100 transgender women *prior* to initiation of gender-affirming hormone treatment, the median total motile sperm count was 0.5 million per vial and less than a quarter of samples were suitable for intrauterine insemination [89]. In fertility preservation counselling for transgender women, this finding, as well as the potential worsening of sperm parameters with tight undergarments and tucking, should be discussed. Individuals electing to undergo sperm cryopreservation should receive timely feedback on the quality of their cryopreserved specimen and recommendations for additional samples.

Embryo cryopreservation

For transfeminine individuals with a partner who is capable and willing to provide oocytes or who feel comfortable selecting an oocyte donor at the time of fertility preservation, embryo cryopreservation represents another option to preserve fertility. Collection of a sperm sample is the same as for those electing to pursue sperm cryopreservation. Again, alternative methods of sperm collection including electroejaculation, aspiration, or

surgical extraction should be discussed. For individuals with diminished sperm parameters, intracytoplasmic sperm injection allows for higher rates of fertilization to be achieved. As for transmasculine people, embryo cryopreservation has the advantages of allowing for genetic testing and improved counselling regarding probability of a live birth from cryopreserved tissue. For individuals who create embryos using their own gametes with a partner's gametes, they may not retain sole ownership over their reproductive tissue and embryo disposition conflicts may arise particularly in the setting of changes in relationship status.

Testicular tissue cryopreservation

Testicular tissue cryopreservation is a method of fertility preservation in which testicular tissue is surgically extracted and cryopreserved for future reproductive use. It is currently the only fertility preservation option available for prepubertal transfeminine youth. While there have been significant scientific progress in this technique over the past decade, testicular tissue cryopreservation has not advanced to the same degree of ovarian tissue cryopreservation. At the time of this chapter, there have not been any published clinical trials of autologous transplantation of immature testicular tissue in humans and complete *in vitro* spermatogenesis has not been achieved with human immature testicular tissue [100]. For the TGNB community, testicular tissue cryopreservation does offer a future means to achieve fertility preservation at the time of gender-affirming surgery without a delay in gender transition and with the potential to avoid gender dysphoria associated with providing a sperm sample for cryopreservation.

Reproductive options for transgender and nonbinary individuals desiring genetically related offspring

Reproductive options counselling for TGNB individuals should initiate with discussion of an individual's reproductive gametes and, if they have a uterus, their desire or willingness to carry a pregnancy (Figure 68.1). Providers should refrain from making assumptions of an individual's sexual preferences or family planning desires on the basis of their gender identity, but rather openly discuss fertility options in the setting of the patient and potential partner's reproductive goals. For couples who are biologically capable and willing to conceive naturally this may involve optimization of natural fertility and for others intrauterine insemination may be a preferred route to conceive. For TGNB individuals who are unpartnered or whose partner is unwilling or unable to produce reproductive gametes, discussion should include third-party reproduction options in the setting of intrauterine insemination or *in vitro* fertilization. TGNB individuals seeking to build families may face unique challenges, including possible discontinuation of gender-affirming hormone treatment, exacerbation of gender dysphoria, consideration for third-party reproduction, and disclosure of their gender identity to future children. A qualified mental health professional should be included in the care team in order to assist with these additional challenges. Given the potential for gender dysphoria associated with fertility treatments, clinics should prioritize a welcoming and inclusive environment with consideration for potential modifications to improve patient comfort. If a patient's anatomy allows, a transabdominal or transrectal ultrasound may be considered as an alternative approach to pelvic ultrasound monitoring, or paediatric

TABLE 68.3 Fertility Preservation Options for Transfeminine Individuals with Testes

Technique	Means to Achieve Parenthood	Advantages	Disadvantages	Strategies to Reduce gender Dysphoria
Sperm Cryopreservation	- future intrauterine insemination in a partner with a uterus - future fertilization of partner oocytes or donor oocytes with resultant embryo transferred to a partner with a uterus or a gestational carrier	- minimally invasive for individuals able to provide an ejaculatory sample - unlikely to delay gender transition - relatively low cost - may be possible even in individuals that have initiated gender-affirming medical treatment - does not require partner or donor at time of preservation - individual ownership over reproductive gametes	- prior hormone treatment or gender dysphoria may limit ability to provide an ejaculatory sample - decreased sperm parameters found in studies of hormone naïve transgender women	- offer collection at home when possible - consider alternative options to specimen collection including electroejaculation or testicular biopsy
Embryo Cryopreservation	- fertilization of partner oocytes or donor oocytes with resultant embryo transferred to a partner with a uterus or a gestational carrier	- allows for genetic testing of embryo for aneuploidy and/or inherited disorder - if partner has ovaries allows for both partners to preserve gametes together - increased ability to predict probability of a live birth resulting from cryopreserved tissue	- requires partner with ovaries or donor selection at time of preservation - if using partner oocytes may no longer have individual ownership over reproductive material	- same considerations as sperm cryopreservation
Testicular Tissue Cryopreservation	- dependent upon future advances to allow <i>in vitro</i> spermatogenesis or auto-transplantation of cryopreserved testicular tissue	- only fertility preservation option available to prepubertal individuals - individual ownership over reproductive tissue	- requires surgical extraction of tissue - promising results in animal studies but lack of auto-transplantation pilot trials in humans and complete spermatogenesis not yet achieved with human immature testicular tissue - future autologous transplant may be gender dysphoric	- may be performed at the same time as gender-affirming surgery - consultation with qualified mental health professional

probes may be utilized to limit discomfort and reduce gender dysphoria. Providers should additionally be aware of the high rates of sexual assault among TGNB individuals and consider a trauma-based approach to pelvic exams or invasive testing.

ART outcomes for TGNB individuals

Qualitative studies of TGNB individuals undergoing fertility treatments show a diversity of experience with insights into challenges faced by this community and potential opportunities to reduce gender dysphoria. In one study of the experiences of 11 transgender individuals with ART, the majority of people reported an overall negative experience citing problems with clinical documentation, misgendering, and cisnormative or heteronormative assumptions of providers as contributing to their overall discomfort. Of those who reported the experience to be overall positive, a welcoming clinic environment and use of gender-neutral terminology caused them to feel more

comfortable [21]. In another study of transgender men undergoing oocyte cryopreservation, certain aspects of treatment, including discontinuation of testosterone with resumption of menses, body changes with gonadotropin stimulation, repeat pelvic ultrasounds, and failure to use gender-neutral terminology, contributed to gender dysphoria [45]. These qualitative studies highlight the need for inclusivity and higher standards for gender-affirming fertility care. Intake forms and educational materials should be designed to be affirming to TGNB individuals and care teams need to avoid assumptions of sexual orientation based on gender identity and prioritize using preferred pronouns and gender-neutral terminology.

Current studies of ART in TGNB individuals show promising results, but are limited in size and the ability to assess long-term outcomes for patients and their offspring. In a recent retrospective cohort of 12 transgender men undergoing ovarian stimulation, half of whom had prior testosterone treatment, an excellent response was observed with no difference in oocytes retrieved or

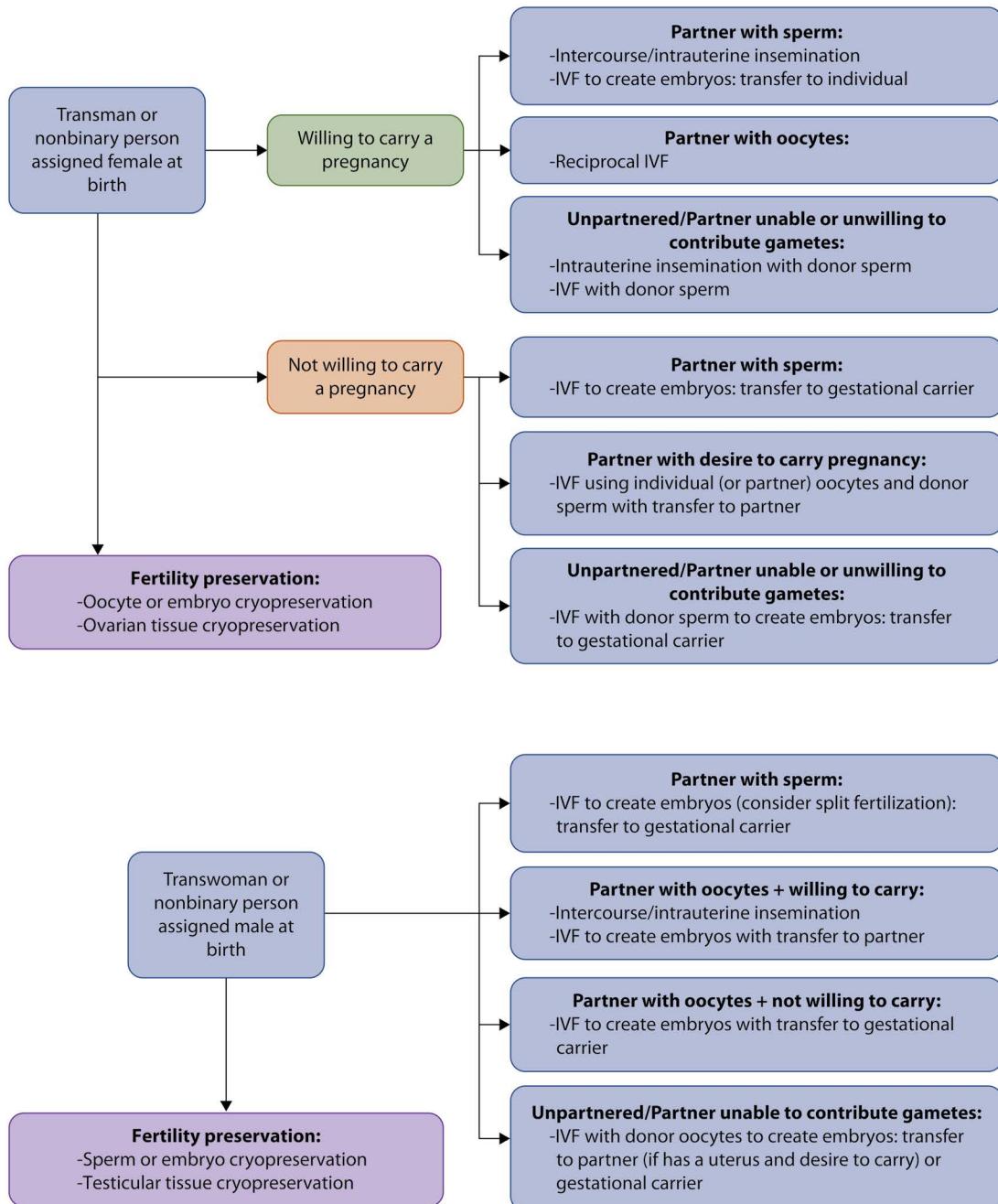


FIGURE 68.1 Reproductive options for transgender and nonbinary individuals.

oocyte maturity as compared to cisgender oocyte donors. Five of the transgender men with prior testosterone treatment underwent embryo cryopreservation and all achieved good-quality embryos [101]. In another cohort of 13 transgender men, seven of which had prior testosterone treatment, compared to age and BMI-matched cisgender controls, again no difference was found between total or mature oocytes. However, transgender men with prior testosterone treatment had fewer oocytes retrieved as compared to those without prior hormone treatment [102]. A third cohort of 26 transgender men undergoing ovarian stimulation, the majority of whom had prior testosterone treatment which was discontinued prior to stimulation, showed excellent outcomes

with a mean of 20 oocytes retrieved. Although transgender men with prior testosterone exposure had higher total gonadotropin use, there was no difference in total or mature oocytes retrieved as compared to matched cisgender females. All seven couples who transferred embryos in this cohort eventually achieved a successful pregnancy [103]. More recently, the first case report of two transgender men continuing gender-affirming testosterone treatment during ovarian stimulation was published, which illustrated that ovarian stimulation is feasible without testosterone discontinuation as 30 and 9 mature oocytes were cryopreserved, respectively [104]. A recent investigation of assisted reproductive technology outcomes from transgender men with prior

testosterone treatment compared with embryos from cisgender women found no differences in fertilization rates or early embryo development between groups [105].

While these studies support the feasibility of oocyte and embryo cryopreservation in transgender women, many questions remain unanswered. The impact of testosterone on IVF outcomes is largely unknown as well as whether this potential impact is partially or fully reversible. There are currently no standard practice guidelines for ovarian stimulation protocols in TGNB individuals, and a key point of uncertainty is whether individuals who have initiated testosterone treatment need to discontinue treatment to pursue oocyte or embryo cryopreservation. Some reproductive endocrinologists recommend a wash-out period of one to three months for transgender individuals on testosterone prior to pursuing fertility preservation, which can be prohibitive particularly for those with severe gender dysphoria. In one survey of transgender and gender diverse youth, only 3% of respondents were willing to delay hormone treatment up to three months for fertility preservation but over a third would choose to undergo fertility preservation if it was possible to preserve fertility while taking hormones [48]. Given the lack of data, it is reasonable to consider testosterone discontinuation on a case-by-case basis using assessment of baseline ovarian reserve in conjunction with risk and benefits of treatment discontinuation to guide decisions in a patient-centred model. Additional strategies reproductive endocrinologists may consider for TGNB individuals are use of a random-start protocol to minimize delay of gender transition and inclusion of an aromatase inhibitor such as letrozole during ovarian stimulation to limit the rise of oestradiol [104–107].

Additional considerations for TGNB youth

TGNB youth face additional challenges when it comes to fertility preservation. The onset of puberty can heighten feelings of gender dysphoria for TGNB youth and it can be difficult to conceptualize future family-building desires at a young age and often in the setting of mental health challenges. Pursuing gender-affirming or fertility preservation treatments at a minority age requires parental onset, which can present an added barrier. Additionally, current fertility preservation options for prepubertal TGNB individuals are limited to ovarian and testicular tissue cryopreservation. There is limited literature regarding the experience of TGNB adolescents with fertility preservation. One qualitative study of a transgender adolescent's experience with oocyte cryopreservation highlights that even for an adolescent who is confident in their desire to preserve fertility and satisfied with their choice, the invasiveness and gender dysphoria associated with the process make it challenging [108].

Pubertal suppression

For young TGNB individuals with persistent gender dysphoria whose pubertal development has reached Tanner stage 2, pubertal suppression is the standard of care. Pubertal suppression, accomplished with GnRH agonist treatment, is a reversible treatment which prevents development of secondary sex characteristics of one's natal gender. It allows TGNB youth and their families a means to halt pubertal development and its associated changes likely to worsen gender dysphoria and provides time to determine whether future gender-affirming medical or surgical treatments are desired. TGNB individuals interested in pubertal suppression should receive fertility preservation counselling prior to

initiation of treatment including discussion of the lack of data regarding long-term fertility outcomes. Studies of transgender youth receiving pubertal suppression have shown improvement in mental health and lower lifetime risk of suicidal ideation [109, 110]. Additionally, suppression of puberty at Tanner stage 2 can prevent irreversible pubertal changes such as vocal masculinization, skeletal changes, and facial feature development, which can be costly and sometimes challenging to try to correct with future gender-affirming surgical treatments [111].

Fertility preservation outcomes with pubertal suppression

Knowledge of the impact of pubertal suppression on fertility preservation is limited to a few case reports. In the first case study of ovarian stimulation in a transmasculine adolescent with a GnRH implant in place the outcome was disappointing with only four mature oocytes retrieved [112]. However, another case study of a transmasculine adolescent with a histrelin implant in place for years prior to ovarian stimulation had greater success with 15 oocytes retrieved, 10 of which were mature [113]. In a third case report of a transmasculine adolescent pursuing oocyte cryopreservation after previous pubertal suppression with a histrelin implant, the implant was removed prior to ovarian stimulation and the patient given Lupron prior to an antagonist protocol with adjunct letrozole. In this case, 22 mature oocytes were retrieved with minimal increase in chest size during stimulation, which resolved with initiation of testosterone [107]. This limited evidence supports the feasibility of ovarian stimulation during or following pubertal suppression with further investigation needed to determine protocol optimization, whether removal of pubertal suppression improves outcomes and the impact of pubertal suppression followed by testosterone treatment on ovarian stimulation.

Conclusion

TGNB individuals represent an underserved population in medicine and reproductive health. They face significant barriers to fertility care, and continued advocacy and research are needed to improve access and fertility experiences for TGNB people. While gender-affirming surgeries have a clear-cut impact on future reproductive potential, current literature on gender-affirming hormone treatment indicate a potential detrimental impact on reproductive capacity with uncertain reversibility. Although there is consensus among major medical societies that universal fertility preservation is standard of care for TGNB individuals prior to gender-affirming treatment, this standard is not being met as counselling is inconsistent and often incomplete. Reproductive options for TGNB individuals depend upon their natal reproductive organs and, if partnered, the reproductive organs of their partner as well as the desire of each to use them. As gender identity does not dictate sexual identity or indicate one's desire to carry a pregnancy, providers should refrain from making assumptions regarding an individual's reproductive goals or desired pathway to parenthood. Although limited by study size and a lack of long-term or offspring outcomes, current literature shows promising results for fertility preservation and reproductive treatments for TGNB individuals. Continued research, including larger collaborative studies, are needed in order to improve understanding of the impact of gender-affirming care on reproductive potential and to optimize fertility treatments for TGNB individuals.

References

1. Flores AR, Herman JL, Gates GL, Brown TN. How Many Adults Identify as Transgender in the United States? The Williams Institute. 2016.
2. Zhang Q, Goodman M, Adams N, Corneil T, Hashemi L, Kreukels B, et al. Epidemiological considerations in transgender health: A systematic review with focus on higher quality data. *Int J Transgend Health.* 2020;21(2):125–37.
3. Winter S, Diamond M, Green J, Karasic D, Reed T, Whittle S, et al. Transgender people: Health at the margins of society. *Lancet.* 2016 Jul 23;388(10042):390–400.
4. Office of Disease Prevention and Health promotion. LGBT. Healthy People 2030. U.S. Department of Health and Human Service. [Internet]. [cited 2022 Mar 2]. Available from: <https://health.gov/healthypeople/objectives-and-data/browse-objectives/lgbt>
5. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders (5th Edition). Fifth. 2013. 947 p.
6. Grant JM, Mottet LA, Tanis J. Injustice at Every Turn: A Report of the National Transgender Discrimination Survey. Washington, DC: National Center for Transgender Equality and National Gay and Lesbian Task Force, 2011.
7. Stotzer RL, Herman JL, Hasenbush A. Transgender Parenting: A Review of Existing Research. Los Angeles, CA: Williams Institute, 2014.
8. Pyne J, Bauer G, Bradley K. Transphobia and other stressors impacting trans parents. *J GLBT Fam Stud.* 2015;11(2):107–26.
9. Carone N, Rothblum ED, Bos HMW, Gartrell NK, Herman JL. Demographics and health outcomes in a U.S. probability sample of transgender parents. *J Fam Psychol.* 2021;35(1):57–68.
10. Auer MK, Fuss J, Nieder TO, Briken P, Biedermann S, Stalla GK, et al. Desire to have children among transgender people in Germany: A cross-sectional multi-center study. *J Sex Med.* 2018; 15(5):757–67.
11. Defreyne J, van Schuylbergh J, Motmans J, Tillemans KL, T'Sjoen GGR. Parental desire and fertility preservation in assigned female at birth transgender people living in Belgium. *Fertil Steril.* 2020;113(1):149–57.e2.
12. Wierckx K, van Caenegem E, Pennings G, Elaut E, Dedecker D, van de Peer E, et al. Reproductive wish in transsexual men. *Hum Reprod.* 2012 Feb;27(2):483–7.
13. Baram S, Myers SA, Yee S, Librach CL. Fertility preservation for transgender adolescents and young adults: A systematic review. *Hum Reprod Update.* 2019;25(6):694–716.
14. Tornello SL, Bos H. Parenting intentions among transgender individuals. *LGBT Health.* 2017;4(2):115–20.
15. Ethics Committee of the American Society for Reproductive Medicine. Access to fertility services by transgender and nonbinary persons: An ethics committee opinion. *Fertil Steril.* 2021 Apr;115(4):874–8.
16. Jones HW. Gender reassignment and assisted reproduction. Evaluation of multiple aspects. *Hum Reprod.* 2000;15(5):987.
17. White T, Ettner R. Adaptation and adjustment in children of transsexual parents. *Eur Child Adolesc Psychiatry.* 2007;16(4):215–21.
18. Chiland C, Clouet A-M, Golse B, Guinot M, Wolf JP. A new type of family: Transmen as fathers thanks to donor sperm insemination. A 12-year follow-up exploratory study of their children. *Neuropsychiatre de l'Enfance et de l'Adolescence.* 2013;61(6):365–70.
19. Condat A, Mamou G, Lagrange C, Mendes N, Wielart J, Poirier F, et al. Transgender fathering: Children's psychological and family outcomes. *PLOS ONE.* 2020 Nov 19;15(11):e0241214.
20. American Academy of Child & Adolescent Psychiatry. Gay, Lesbian, Bisexual, or Transgender Parents Policy Statement (revised 2009). [cited 2022 Feb 2]; Available from: https://www.aacap.org/AACAP/Policy_Statements/2008/Gay_Lesbian_Bisexual_or_Transgender_Parents.aspx
21. James-Abra S, Tarasoff LA, green d, Epstein R, Anderson S, Marvel S, et al. Trans people's experiences with assisted reproduction services: A qualitative study. *Hum Reprod.* 2015 Jun 1;30(6):1365–74.
22. de Wert G, Dondorp W, Shenfield F, Barri P, Devroey P, Diedrich K, et al. ESHRE task force on ethics and law 23: Medically assisted reproduction in singles, lesbian and gay couples, and transsexual people. *Hum Reprod.* 2014 Sep 1;29(9):1859–65.
23. James SE, Herman JL, Rankin S, Keisling M, Mottet L, Anafi M. Executive Summary of the Report of the 2015 U.S. Transgender Survey. Washington, DC: National Center for Transgender Equality, 2015.
24. SFS 1972:119: Lag (1972:119); see also, J Garland, 'The legal status of transsexual and transgender persons in Sweden' in JM Scherpe (eds.). *The Legal Status of Transsexual and Transgender Persons.* United Kingdom and Belgium: Intersentia, 2015, 281.
25. Dunne P. Transgender sterilisation requirements in Europe. *Medical Law Review.* 2017;25(4):554–81.
26. European Court of Human Rights, Garcon AP, France Nicot v. Nos 79885/12, 5247/13, 52596/13, ECHR 2017, Judgement of 6 April 2017, para 131 (French version).
27. Trans Rights Europe & Central Asia Map & Index 2019 [Internet]. [cited 2022 Mar 2]. Available from: <https://tgeu.org/trans-rights-europe-central-asia-map-index-2019/>
28. Cisek v Visek, No 80 C.A. 113, 1982 WL 6161 at 1-2 [Ohio Ct, App. July 20, 1982].
29. Magnuson v Magnuson, 170 P.3d 65, 66 [Wash. Ct. App. 2007].
30. M.B. v. D.W., 236 S.W.3d 31 [Ky. Ct. App. 2007].
31. Farr RH, Goldberg AE. Sexual orientation, gender identity, and adoption law. *Family Court Review.* 2018;56(3):374–83.
32. Cooper L. Protecting the rights of transgender parents and their children a guide for parents and lawyers. American Civil Liberties Union [Internet]. 2013 [cited 2022 Feb 1]; Available from: https://www.aclu.org/sites/default/files/field_document/aclu-tg_parenting_guide.pdf
33. Katyal S, Turner IM. Transparency. *SSRN Electronic Journal.* 2019.
34. European Union Agency for Fundamental Rights. Being Trans in the EU - Comparative analysis of the EU LGBT survey data. 2014.
35. Kosenko K, Rintamaki L, Raney S, Maness K. Transgender patient perceptions of stigma in health care contexts. *Medical Care.* 2013;51(9):819–22.
36. Obedin-Maliver J, Goldsmith ES, Stewart L, White W, Tran E, Brennan S, et al. Lesbian, gay, bisexual, and Transgender–Related content in undergraduate medical education. *JAMA.* 2011 Sep 7;306(9).
37. Shires DA, Stroumsa D, Jaffee KD, Woodford MR. Primary care Clinicians' willingness to care for transgender patients. *Ann Fam Med.* 2018 Nov 12;16(6):555–8.
38. Unger CA. Care of the transgender patient: A survey of Gynecologists' current knowledge and practice. *J Women Health.* 2015;24(2):114–8.
39. Irwig MS. Transgender care by endocrinologists in the United States. *Endocr Pract.* 2016;22(7):832–6.
40. Davidge-Pitts CJ, Nippoldt TB, Natt N. Endocrinology fellows' perception of their confidence and skill level in providing transgender healthcare. *Endocr Pract.* 2018;24(12):1038–42.
41. Health and Human Services Department. Nondiscrimination in health programs and activities. [cited 2022 Mar 2]; Available from: <http://www.federalregister.gov/d/2016-11458>
42. Vyas N, Douglas CR, Mann C, Weimer AK, Quinn MM. Access, barriers, and decisional regret in pursuit of fertility preservation among transgender and gender-diverse individuals. *Fertil Steril.* 2021;115(4):1029–34.
43. Learmonth C, Viloria R, Lambert C, Goldhammer H, Keuroghlian AS. Barriers to insurance coverage for transgender patients. *Am J Obstetr Gynecol.* 2018;219(3):272.e1–4.
44. Lai TC, McDougall R, Feldman D, Elder C, Pang KC. Fertility counseling for transgender adolescents: A review. *J Adolesc Health.* 2020;66(6):658–65.

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45. Armuand G, Dhejne C, Olofsson JI, Rodriguez-Wallberg KA. Transgender men's experiences of fertility preservation: A qualitative study. *Hum Reprod.* 2017;32(2):383–90.
46. Nahata L, Tishelman AC, Caltabellotta NM, Quinn GP. Low fertility preservation utilization among transgender youth. *J Adolesc Health.* 2017;61(1):40–4.
47. Briik T, Vrouenraets LJ, Schagen SEE, Meissner A, de Vries MC, Hannema SE. Use of fertility preservation among a cohort of transgirls in the Netherlands. *J Adolesc Health.* 2019;64(5): 589–93.
48. Persky RW, Gruschow SM, Sinai N, Carlson C, Ginsberg JP, Dowshen NL. Attitudes toward fertility preservation among transgender youth and their parents. *J Adolesc Health.* 2020;67(4): 583–9.
49. MacDonald T, Noel-Weiss J, West D, Walks M, Biener M, Kibbe A, et al. Transmasculine individuals' experiences with lactation, chestfeeding, and gender identity: A qualitative study. *BMC Pregnancy Childbirth.* 2016 Dec 16;16(1):106.
50. Meyer G, Mayer M, Mondorf A, Flügel AK, Herrmann E, Bojunga J. Safety And rapid efficacy of guideline-based gender-affirming hormone therapy: An Analysis of 388 individuals diagnosed with gender dysphoria. *Eur J Endocrinol.* 2020;182(2):149–56.
51. Nakamura A, Watanabe M, Sugimoto M, Sako T, Mahmood S, Kaku H, et al. Dose-response analysis of testosterone replacement therapy in patients with female to male gender identity disorder. *Endocr J.* 2013;60(3):275–81.
52. Spratt DI, Stewart II, Savage C, Craig W, Spack NP, Chandler DW, et al. Subcutaneous injection of testosterone is an effective and preferred alternative to intramuscular injection: Demonstration in female-to-male transgender patients. *J Clin Endocrinol Metab.* 2017 Jul 1;102(7):2349–55.
53. Olson J, Schrager SM, Clark LF, Dunlap SL, Belzer M. Subcutaneous testosterone: An effective delivery mechanism for masculinizing young transgender men. *LGBT Health.* 2014;1(3):165–7.
54. Defreyne J, Vanwonterghem Y, Collet S, Iwamoto SJ, Wiepjes CM, Fisher AD, et al. Vaginal bleeding and spotting in transgender men after initiation of testosterone therapy: A prospective cohort study (ENIGI). *Int J Transgend Health.* 2020 Apr 2;21(2):163–75.
55. Light A, Wang L-F, Zeymo A, Gomez-Lobo V. Family planning and contraception use in transgender men. *Contraception.* 2018;98(4):266–9.
56. Light AD, Obedin-Maliver J, Sevelius JM, Kerns JL. Transgender men who experienced pregnancy after female-to-male gender transitioning. *Obstetr Gynecol.* 2014;124(6):1120–7.
57. Cao CD, Amero MA, Marcinkowski KA, Rosenblum NG, Chan JSY, Richard SD. Clinical characteristics and histologic features of hysterectomy specimens from transmasculine individuals. *Obstetr Gynecol.* 2021;138(1):51–7.
58. Grynberg M, Fanchin R, Dubost G, Colau J-C, Brémont-Weil C, Frydman R, et al. Histology of genital tract and breast tissue after long-term testosterone administration in a female-to-male transsexual population. *Reprod Biomed Online.* 2010 Apr;20(4):553–8.
59. Hawkins M, Deutsch MB, Obedin-Maliver J, Stark B, Grubman J, Jacoby A, et al. Endometrial findings among transgender and gender nonbinary people using testosterone at the time of gender affirming hysterectomy. *Fertil Steril.* 2021 May;115(5):1312–7.
60. Grimstad FW, Fowler KG, New EP, Ferrando CA, Pollard RR, Chapman G, et al. Uterine pathology in transmasculine persons on testosterone: A retrospective multicenter case series. *Am J Obstetr Gynecol.* 2019 Mar;220(3):257.e1–257.e7.
61. Perrone AM, Cerpolini S, Maria Salvi NC, Ceccarelli C, de Giorgi LB, Formelli G, et al. Effect of long-term testosterone administration on the endometrium of female-to-male (FtM) transsexuals. *J Sex Med.* 2009 Nov;6(11):3193–200.
62. Kinnear HM, Constance ES, David A, Marsh EE, Padmanabhan V, Shikanov A, et al. A mouse model to investigate the impact of testosterone therapy on reproduction in transgender men. *Hum Reprod.* 2019 Oct 2;34(10):2009–17.
63. van den Broecke R, van der Elst J, Liu J, Hovatta O, Dhont M. The female-to-male transsexual patient: A source of human ovarian cortical tissue for experimental use. *Hum Reprod.* 2001;16(1):145–7.
64. Ikeda K, Baba T, Noguchi H, Nagasawa K, Endo T, Kiya T, et al. Excessive androgen exposure in female-to-male transsexual persons of reproductive age induces hyperplasia of the ovarian cortex and stroma but not polycystic ovary morphology. *Hum Reprod.* 2013 Feb 1;28(2):453–61.
65. Grimstad FW, Fowler KG, New EP, Ferrando CA, Pollard RR, Chapman G, et al. Ovarian histopathology in transmasculine persons on testosterone: A multicenter case series. *J Sex Med.* 2020 Sep;17(9):1807–18.
66. Marschalek J, Pietrowski D, Dekan S, Marschalek M-L, Brandstetter M, Ott J. Markers of vitality in ovaries of transmen after long-term androgen treatment: A prospective cohort study. *Mol Med.* 2020;26(1):83.
67. Loverro G, Resta L, Dellino M, Edoardo DN, Cascarano MA, Loverro M, et al. Uterine and ovarian changes during testosterone administration in young female-to-male transsexuals. *Taiwan J Obstetr Gynecol.* 2016 Oct;55(5):686–91.
68. Caanen MR, Soleman RS, Kuijper EAM, Kreukels BPC, de Roo C, Tillemen K, et al. Antimüllerian hormone levels decrease in female-to-male transsexuals using testosterone as cross-sex therapy. *Fertil Steril.* 2015 May;103(5):1340–5.
69. Tack LJW, Craen M, Dhondt K, vanden Bossche H, Laridaen J, Cools M. Consecutive lynestrenol and cross-sex hormone treatment in biological female adolescents with gender dysphoria: A retrospective analysis. *Biol Sex Differ.* 2016 Dec 16;7(1):14.
70. Yaish I, Tordjman K, Amir H, Malinger G, Salemkick Y, Shefer G, et al. Functional ovarian reserve in transgender men receiving testosterone therapy: Evidence for preserved anti-Müllerian hormone and antral follicle count under prolonged treatment. *Hum Reprod.* 2021 Sep 18;36(10):2753–60.
71. Bartels CB, Uliasz TF, Lestz L, Mehlmann LM. Short-term testosterone use in female mice does not impair fertilizability of eggs: Implications for the fertility care of transgender males. *Hum Reprod.* 2021;36(1):189–98.
72. Rothenberg SS, Steimer S, Munyoki S, Sheng Y, Sukhwani M, Valli-Pulaski H, et al. The effect of masculinizing therapies on ART outcomes in female mice. *Fertil Steril.* 2019 Apr;111(4):e13.
73. de Roo C, Lierman S, Tillemen K, Peynshaert K, Braeckmans K, Caanen M, et al. Ovarian tissue cryopreservation in female-to-male transgender people: Insights into ovarian histology and physiology after prolonged androgen treatment. *Reprod Biomed Online.* 2017 Jun;34(6):557–66.
74. Lierman S, Tillemen K, Braeckmans K, Peynshaert K, Weyers S, T'Sjoen G, et al. Fertility preservation for trans men: Frozen-thawed in vitro matured oocytes collected at the time of ovarian tissue processing exhibit normal meiotic spindles. *J Assist Reprod Genet.* 2017 Nov 24;34(11):1449–56.
75. Lierman S, Tolpe A, de Croo I, de Gheselle S, Defreyne J, Baetens M, et al. Low feasibility of in vitro matured oocytes originating from cumulus complexes found during ovarian tissue preparation at the moment of gender confirmation surgery and during testosterone treatment for fertility preservation in transgender men. *Fertil Steril.* 2021 Oct;116(4):1068–76.
76. Schneider F, Neuhaus N, Wistuba J, Zitzmann M, Heß J, Mahler D, et al. Testicular functions and clinical characterization of patients with gender dysphoria (GD) undergoing sex reassignment surgery (SRS). *J Sex Med.* 2015;12(11):2190–200.
77. Matoso A, Khandakar B, Yuan S, Wu T, Wang LJ, Lombardo KA, et al. Spectrum of findings in orchectomy specimens of persons undergoing gender confirmation surgery. *Hum Pathol.* 2018; 76:91–9.
78. Kent MA, Winoker JS, Grotas AB. Effects of feminizing hormones on sperm production and malignant changes: Microscopic examination of post orchectomy specimens in transwomen. *Urology.* 2018;121:93–6.

79. Sinha A, Mei L, Ferrando C. The effect of estrogen therapy on spermatogenesis in transgender women. *F S Rep.* 2021;2(3):347–51.
80. Vereecke G, Defreyne J, van Saen D, Collet S, van Dorpe J, T'Sjoen G, et al. Characterisation of testicular function and spermatogenesis in transgender women. *Hum Reprod.* 2021;36(1):5–15.
81. de Nie I, Mulder CL, Meißner A, Schut Y, Holleman EM, van der Sluis WB, et al. Histological study on the influence of puberty suppression and hormonal treatment on developing germ cells in transgender women. *Hum Reprod.* 2022 Jan 28;37(2):297–308.
82. Jiang DD, Swenson E, Mason M, Turner KR, Dugi DD, Hedges JC, et al. Effects of estrogen on spermatogenesis in transgender women. *Urology.* 2019;132:117–22.
83. Leavy M, Trottmann M, Liedl B, Reese S, Stief C, Freitag B, et al. Effects of elevated β -estradiol levels on the functional morphology of the testis - new insights. *Sci Rep.* 2017 Feb 3;7(1):39931.
84. Lübbert H, Leo-Roßberg I, Hammerstein J. Effects of ethinyl estradiol on semen quality and various hormonal parameters in a eugonal male. *Fertil Steril.* 1992;58(3):603–8.
85. Mohammadzadeh M, Pourentezi M, Zare-Zardini H, Nabi A, Esmailabad SG, Khodadadian A, et al. The effects of sesame oil and different doses of estradiol on testicular structure, sperm parameters, and chromatin integrity in old mice. *Clin Exp Reprod Med.* 2021 Mar 1;48(1):34–42.
86. Adeleye AJ, Reid G, Kao C-N, Mok-Lin E, Smith JF. Semen parameters among transgender women with a history of hormonal treatment. *Urology.* 2019;124:136–41.
87. Rodriguez-Wallberg KA, Häljestig J, Arver S, Johansson AL, Lundberg FE. Sperm quality in transgender women before or after gender affirming hormone therapy—A prospective cohort study. *Andrology.* 2021 Nov 23;9(6):1773–80.
88. de Nie I, Meißner A, Kosteljik EH, Soufan AT, Voorn-de Warem IAC, den Heijer M, et al. Impaired semen quality in trans women: Prevalence and determinants. *Hum Reprod.* 2020 Jul 1;35(7):1529–36.
89. de Nie I, Asseler J, Meißner A, Voorn-de Warem IAC, Kosteljik EH, den Heijer M, et al. A cohort study on factors impairing semen quality in transgender women. *Am J Obstetr Gynecol.* 2022 Mar;226(3):390.e1–e10.
90. Hamada A, Kingsberg S, Wierckx K, T'Sjoen G, de Sutter P, Knudson G, et al. Semen characteristics of transwomen referred for sperm banking before sex transition: A case series. *Andrologia.* 2015 Sep;47(7):832–8.
91. Li K, Rodriguez D, Gabrielsen JS, Centola GM, Tanrikut C. Sperm cryopreservation of transgender individuals: Trends and findings in the past decade. *Andrology.* 2018;6(6):860–4.
92. World Professional Association for Transgender Health. Standards of Care for the Health of Transsexual, Transgender, and Gender-Conforming People. 2012 [cited 2022 Feb 1]; Available from: <https://www.wpath.org/publications/soc>.
93. Hembree WC, Cohen-Kettenis PT, Gooren L, Hannema SE, Meyer WJ, Murad MH, et al. Endocrine treatment of gender-dysphoric/gender-incongruent persons: An endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* 2017 Nov 1;102(11):3869–903.
94. Riggs DW, Bartholomaeus C. Fertility preservation decision making amongst Australian transgender and non-binary adults. *Reprod Health.* 2018 Dec 25;15(1):181.
95. Chiniara LN, Viner C, Palmert M, Bonifacio H. Perspectives on fertility preservation and parenthood among transgender youth and their parents. *Arch Dis Child.* 2019;104(8):739–44.
96. Reichman DE, Davis OK, Zaninovic N, Rosenwaks Z, Goldschlag DE. Fertility preservation using controlled ovarian hyperstimulation and oocyte cryopreservation in a premenarcheal female with myelodysplastic syndrome. *Fertil Steril.* 2012;98(5):1225–8.
97. Azem F, Brener A, Malinger G, Reches A, Many A, Yogeve Y, et al. Bypassing physiological puberty, A novel procedure of oocyte cryopreservation at age 7: A case report and review of the literature. *Fertil Steril.* 2020 Aug;114(2):374–8.
98. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: A committee opinion. *Fertil Steril.* 2019 Dec;112(6):1022–33.
99. Donnez J, Dolmans M-M. Fertility preservation in women. *N Engl J Med.* 2017 Oct 26;377(17):1657–65.
100. Wyns C, Kanbar M, Giudice MG, Poels J. Fertility preservation for prepubescent boys: Lessons learned from the past and update on remaining challenges towards clinical translation. *Hum Reprod Update.* 2021 Apr 21;27(3):433–59.
101. Amir H, Yaish I, Samara N, Hasson J, Groutz A, Azem F. Ovarian stimulation outcomes among transgender men compared with fertile cisgender women. *J Assist Reprod Genet.* 2020 Oct 28;37(10):2463–72.
102. Adeleye AJ, Cedars MI, Smith J, Mok-Lin E. Ovarian stimulation for fertility preservation or family building in a cohort of transgender men. *J Assist Reprod Genet.* 2019 Oct 21;36(10):2155–61.
103. Leung A, Sakkas D, Pang S, Thornton K, Resetkova N. Assisted reproductive technology outcomes in female-to-male transgender patients compared with cisgender patients: A new frontier in reproductive medicine. *Fertil Steril.* 2019;112(5):858–65.
104. Stark BA, Mok-Lin E. Fertility preservation in transgender men without discontinuation of testosterone. *FS Rep.* 2022 Feb;3(2):153–6.
105. Israeli T, Preisler L, Kalma Y, Groutz A, Azem F, Amir H. Similar fertilization rates and preimplantation embryo development among testosterone-treated transgender men and cisgender women. *Reprod Biomed Online.* 2022 Apr;45(3):448–56.
106. Cho K, Harjee R, Roberts J, Dunne C. Fertility preservation in a transgender man without prolonged discontinuation of testosterone: A case report and literature review. *FS Rep.* 2020;1(1):43–7.
107. Martin CE, Lewis C, Omurtag K. Successful oocyte cryopreservation using letrozole as an adjunct to stimulation in a transgender adolescent after GnRH agonist suppression. *Fertil Steril.* 2021;116(2):522–7.
108. Chen D, Simons L. Ethical considerations in fertility preservation for transgender youth: A case illustration. *Clin Pract Pediatr Psychol.* 2018;6(1):93–100.
109. de Vries ALC, McGuire JK, Steensma TD, Wagenaar ECF, Doreleijers TAH, Cohen-Kettenis PT. Young adult psychological outcome after puberty suppression and gender reassignment. *Pediatrics.* 2014;134(4):696–704.
110. Turban JL, King D, Carswell JM, Keuroghlian AS. Pubertal suppression for transgender youth and risk of suicidal ideation. *Pediatrics.* 2020 Feb 1;145(2):e20191725.
111. Panagiotakopoulos L, Chulani V, Koyama A, Childress K, Forcier M, Grimsby G, et al. The effect of early puberty suppression on treatment options and outcomes in transgender patients. *Nat Rev Urol.* 2020 Nov 23;17(11):626–36.
112. Rothenberg SS, Witchel SF, Menke MN. Oocyte cryopreservation in a transgender male adolescent. *New Engl J Med.* 2019 Feb 28;380(9):886–7.
113. Insogna IG, Ginsburg E, Srouji S. Fertility preservation for adolescent transgender male patients: A case series. *J Adolesc Health.* 2020;66(6):750–3.

OVARIAN HYPERSTIMULATION SYNDROME

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Introduction

Ovarian hyperstimulation syndrome (OHSS) is a serious and uncommon iatrogenic complication of assisted reproduction, caused by the use of gonadotrophins administered for controlled ovarian stimulation. However, it has to be mentioned that OHSS is rare without human chorionic gonadotropin (hCG) administration for ovulation triggering. Indeed, hCG, which is structurally and functionally similar to luteinizing hormone (LH), and has a longer half-life than LH (over 24 hours vs approximately 60 minutes for LH), seems to play a key role in the development of OHSS [1, 2]. The exposure of hyperstimulated ovaries to hCG represents a crucial event causing the production of proinflammatory mediators responsible for the clinical features of OHSS. A variety of cytokines, proinflammatory mediators, and angiogenic molecules, such as vascular endothelial growth factor (VEGF), are likely to be involved in the pathogenesis of OHSS. The occurrence of ovarian enlargement with both local and systemic effects of inflammation mediators, including increased vascular permeability and a prothrombotic effect, are responsible for the clinical characteristics of OHSS. Moderate to severe OHSS occurs roughly in 1%–5% of stimulated cycles [3–7]. Nevertheless, the real incidence is extremely difficult to estimate, since a strict consensus on the definition is lacking.

Pathophysiology

Common physiologic events occurring in OHSS include a spectrum of changes such as increased arteriolar vasodilation and capillary permeability in the region surrounding the ovaries and their vasculature. The crux seems to rely on a fine balance between proangiogenic and antiangiogenic factors present in follicular fluid. These alterations result in a phenomenon of fluid shifting from intravascular to extra-vascular spaces [8, 9] leading to a state of electrolyte unbalance called hypovolemic hyponatremia. VEGF appears to be the chief among proinflammatory mediators, being mainly responsible for the development of OHSS, and being involved in follicular growth, corpus luteum function, angiogenesis, and vascular endothelial stimulation [10–12]. Indeed, VEGF mediates the permeability of vascular endothelium in response to hCG, whose systemic levels are reported to positively correlate with the severity of the syndrome [12–14]. However, the pathogenesis of OHSS is also related to the direct and indirect effects mediated by other systemic and local vasoactive substances, such as interleukin-6, interleukin-1 β , angiotensin II, insulin-like growth factor 1, transforming growth factor β , and the renin-angiotensin system [10, 14–18]. Finally, hCG and its analogues, oestrogens, oestradiol, prolactin, histamine, and prostaglandins, have all been implicated in OHSS.

Risk factors predicting OHSS

Any woman undergoing controlled ovarian stimulation with gonadotropins may potentially develop OHSS. However, there is strong evidence describing that some women have a higher risk.

The identification of these patients is a crucial step to avoid OHSS and to decrease the incidence of this syndrome. Literature reports a variety of risk factors which should have to be considered when assessing the risk of developing OHSS.

Demographic factors (age, BMI, ethnicity, reason for infertility)

There is consistent evidence showing that the younger age is associated with a higher risk of OHSS [4]. Moreover, it seems that the majority of OHSS cases occur in women who are less than 35 years old [4, 19–23]. Similarly, a lower BMI seems to correlate with an increased risk of the syndrome [22–24]. On the other hand, several studies failed to replicate the same findings [19, 21, 25, 26]. With regard to the ethnicity, black race has been described as a predictive factor for OHSS [4]. Among infertility causes, ovulation disorders, tubal factor, and idiopathic infertility were all associated with an increased risk of OHSS [4]. Enough evidence is showing a higher OHSS rate in women affected by polycystic ovary syndrome (PCOS) [4, 5, 21, 22, 25, 27].

Ovarian reserve markers (AMH, AFC, inhibin A/B)

Markers of ovarian reserve, especially anti-Müllerian hormone (AMH) and antral follicle count (AFC), have been found to be predictive for OHSS in several studies. This evidence may be useful in clinical practice to correctly plan and choose ovarian stimulation protocols as well as to appropriately counsel patients regarding their risk. Serum AMH levels have been shown to be predictive for OHSS (cut-off value 3.36 ng/mL) [26]. Additionally, it has been reported that serum AMH levels in women who experienced OHSS were sixfold higher than age- and weight-matched controls [28]. Among women with high AMH (>5 ng/mL), those who had extra-high levels of AMH (>10 ng/mL) had significantly higher rates (more than threefold) of OHSS [29]. Similarly, a correlation has been found between AFC and OHSS [19, 30]. A prospective analysis of 1012 first ART cycles described that the risk of OHSS increases from 2.2% into 8.6% when AFC is ≥24 [5]. Finally, to date, a correlation between serum (or follicular) inhibin A or B concentrations and the development of OHSS has not yet been demonstrated [31].

Ovarian stimulation parameters (follicles, oocytes, serum oestradiol levels)

Multiple growing follicles during stimulation, high oestradiol levels, and elevated number of oocytes retrieved may help to identify those patients who are at risk to develop OHSS. Several studies have shown that a high number of growing follicles act as an independent predictor of OHSS [6, 7, 24, 32]. According to one study, a number of growing follicles ≥20 during ovarian stimulation significantly increases the risk of OHSS [32]. However, there is strong evidence that a count of ≥18 follicles having size ≥11 mm diameter the day of triggering is predictive for high risk of severe

OHSS [6, 33]. On the other hand, other data suggest a threshold of 13–15 follicles having a size of ≥10 mm diameter prior to trigger for prediction of moderate OHSS [34, 35]. A prospective cohort study of 624 patients undergoing their first IVF cycle investigated a model to predict the occurrence of OHSS. A multivariate analysis identified the following thresholds with 82% sensitivity and 90% specificity: >25 follicles at oocyte retrieval, >19 large/medium-sized follicles before hCG, and >24 retrieved oocytes [36]. Several other studies supported the positive correlation between the number of collected oocytes and the development of OHSS [1, 4, 18–24, 32, 33]. Finally, serum oestradiol levels were also significantly associated with OHSS [19–26, 37, 38]. Most of these studies indicated that the mean serum oestradiol concentration in patients with OHSS was >3,500 pg/mL. However, it has to be mentioned that serum oestradiol levels >2500 pg/mL are considered an important predictive factor for development of OHSS [6, 39, 40].

Unusual cases of OHSS

Although OHSS represents an iatrogenic complication of IVF cycles, being usually straightforward with history of ovarian stimulation, it is important to mention that it may occur potentially under unexpected conditions. Several cases of mild to severe OHSS have been reported in literature following ovulation induction with clomiphene citrate [41, 42]. On the other hand, OHSS may also be consequent to spontaneous conception especially in multiple or in molar pregnancies and also in association with hypothyroidism, pituitary tumours, as a familial predisposition and mutation in the FSHR gene [43–47].

Clinical features and diagnosis

Considering the progressive comprehension of controlled ovarian stimulation protocols and monitoring, as well as OHSS pathophysiology, the aim of the clinicians involved in ART should be to mitigate or completely avoid the development of this syndrome. Symptoms of OHSS include nausea, vomiting, diarrhoea, self-reported reduction in urine output, abdominal bloating, mild abdominal pain and/or tension, increased ovarian size (Figure 69.1), ascites, hemo-concentration, hypercoagulability, and electrolyte alteration (Table 69.1). In addition, according to a classification by the American Society for Reproductive Medicine (ASRM) [48], symptoms can be categorized by their severity (mild, moderate, or severe) (Table 69.2). Women with OHSS typically present with abdominal distension and discomfort following the trigger used to promote final follicular maturation prior to oocyte retrieval. In addition, there may be a history of an excessive ovarian response to ovarian stimulation; however, the absence of such a history does not exclude a diagnosis of OHSS (Table 69.1). Based on the time of symptoms presentation following the trigger injection, patients can be divided into two groups, named early and late OHSS. With the term “early” is defined that type of OHSS usually presenting in seven days from the trigger injection; this type of OHSS is commonly associated with an excessive ovarian response. The term “late” indicates OHSS occurring 10 or more days after the trigger injection; this is the result of endogenous hCG derived from an early pregnancy. Late OHSS tends to be more prolonged and severe than the early form [22, 49]. However, it should be pointed out that OHSS is a self-limiting condition especially in patients who do not become pregnant with typical resolution of symptoms at the time of the next menstrual period.

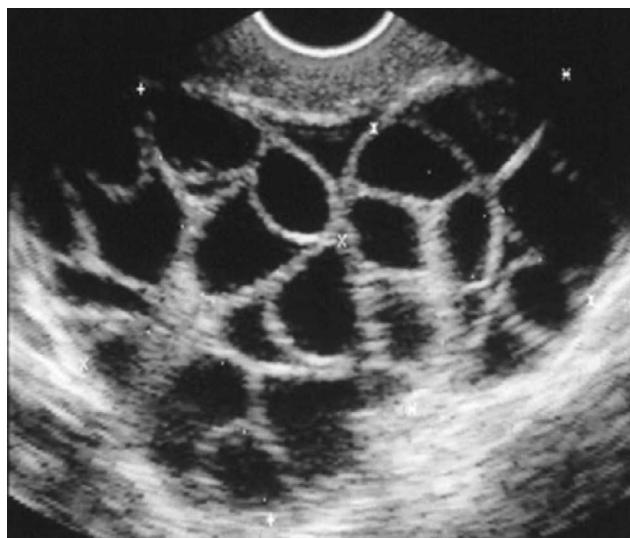


FIGURE 69.1 Hyperstimulated ovaries following ovarian stimulation. (Image adapted from Smith, Laura P. “Ultrasound and ovarian hyperstimulation syndrome.” *Ultrasound Imaging in Reproductive Medicine*. Springer, Cham, 2019. 321–333.)

Conversely, in patients who do conceive, the ovaries continue to be stimulated by the increasing hCG levels with symptoms that may last until the end of the first trimester. Moreover, it has to be mentioned that multiple pregnancies represent a risk factor for the late form due to the higher levels of HCG produced with consequent secretion of higher amounts of vasoactive factors [50]. The symptoms of OHSS are unspecific and, to date, no diagnostic test has been found for this condition. Along this line, it is important to draw special attention to those other serious conditions which may occur with similar clinical manifestations; hence, care is needed to exclude pathologies who are similar in clinic but require different management. Assessment by clinicians may be

TABLE 69.1 Initial Assessment

Questions Regarding the History

- When did the symptoms occur? (Early or late onset relative to trigger)
- Which medication has been used for trigger? (hCG or GnRH agonist)
- How many follicles did you have on final monitoring scan?
- How many oocytes have been collected?
- Were embryos replaced and how many?
- Polycystic ovary syndrome diagnosis?

Symptoms

- Abdominal bloating
- Abdominal discomfort/pain, need for analgesia
- Nausea and vomiting
- Breathlessness, inability to lie flat or talk in full sentences
- Reduced urine output
- Leg swelling
- Vulval swelling
- Associated comorbidities such as thrombosis

TABLE 69.2 Classification of OHSS Symptoms According to the American Society for Reproductive Medicine (ASRM)

Stage	Clinical Features	Blood Alterations
Mild	Abdominal distension/discomfort	No important alterations
	Mild nausea/vomiting	
	Mild dyspnoea	
	Increased measure of the ovaries	
	Diarrhoea	
	Mild stage features	Haemoconcentration (Hct>41%)
Moderate	Ultrasonographic evidence of ascites	Elevated WBC (>15,000 mL)
	Mild and moderate stage features	Severe haemoconcentration (Hct>55%)
Severe	Clinical evidence of ascites	WBC>25,000 mL
	Hydrothorax	CrCl <50 mL/min
	Severe dyspnoea	Cr >1.6 mg/dL
	Oliguria/anuria	Na+ <135 mEq/L
	Intractable nausea/vomiting	K+ >5 mEq/L
	Low blood/central venous pressure	Elevated liver enzymes
	Pleural effusion	
	Rapid weight gain (>1 kg in 24 h)	
	Syncope	
	Anuria/acute renal failure	Worsening of findings
Critical	Arrhythmia	
	Thromboembolism	
	Pericardial effusion	
	Massive hydrothorax	
	Arterial thrombosis	
	Adult respiratory distress syndrome	
	Sepsis	

Source: Adapted from "Practice Committee of the American Society for Reproductive Medicine." Prevention and treatment of moderate and severe ovarian hyperstimulation syndrome: a guideline. *Fertility and sterility.* 2016 Dec 1;106(7):1634–47.

Note: Hct, haematocrit; WBC, white blood cell; Cr, creatinine; CrCl, creatinine clearance.

focused on the evaluation of blood parameters such as full blood count, serum electrolytes, and osmolality. Indeed, reduced serum osmolality and sodium combined with elevated haematocrit is indicative of OHSS [51]. On the other hand, pelvic ultrasound and, eventually, abdominal imaging may be of high clinical relevance to make the diagnosis. Finally, it has to be mentioned that OHSS by itself is rarely associated with severe pain, hyperpyrexia, or peritonism signs. The presence of these features should alert for the investigation of other conditions such as pelvic infections, pelvic abscess, appendicitis, ovarian torsion or cyst rupture, bowel perforation [52], and ectopic pregnancy. For this reason, OHSS should not be considered as a 'default diagnosis' for women presenting with abdominal pain during fertility treatment.

TABLE 69.3 Classification of OHSS according to Golan et al. (1989)

Category	Grade
Mild	Grade 1 Abdominal distension/discomfort
	Grade 2 Features of grade 1 plus nausea, vomiting, and/or diarrhoea
	Ovaries are enlarged to 5–12 cm
Moderate	Grade 3 Features of mild OHSS with ultrasound evidence of ascites
	Grade 4
Severe	Features of moderate OHSS plus clinical evidence of ascites and/or hydrothorax or breathing difficulties
	Grade 5 All of the above plus change in blood volume, increased blood viscosity due to haemoconcentration, coagulation abnormalities, and diminished renal perfusion and function

Classification

According to the OHSS classification proposed by Golan et al. (adopted by the European Society of Human Reproduction and Embryology [ESHRE]) [49, 53], OHSS may be classified according to clinical features: normal—patients reporting no symptoms of OHSS; mild OHSS—patients with at least one symptom consistent with OHSS and maximal ovarian volume (either left or right ovary) of approximated to 5–12 cm diameter; moderate OHSS—patients who met the criteria for mild OHSS with at least 50 mL of total ascitic fluid in the pouch of Douglas, adnexa, and abdomen; severe OHSS—patients who met the criteria for moderate OHSS but having at least 50 mL of fluid in the pleural space (Table 69.3). A revision of this classification [54] showed the addition of a grade 6 defining critical OHSS, as described by Navot et al. (1992) [55], and/or life-threatening complications of OHSS, as suggested by Rizk and Aboulghar (1999) [56] (Table 69.4). Severe forms of OHSS may lead to life-threatening complications, including pleural effusion, acute renal insufficiency, and venous thromboembolism.

TABLE 69.4 New Added Grade 6 to the Classification of OHSS according to Golan et al. (1989)

Complications such as respiratory distress syndrome, renal shut-down, or venous thrombosis
Variably enlarged ovary
Tense ascites ± hydrothorax ± pericardial effusion Haematocrit >55% WBC ≥25,000 cells/mm ³
Oligo/anuria
Creatinine ≥1.6 mg/dL
Creatinine clearance <50 mL/min.
Renal failure
Thromboembolic phenomena
Adult respiratory distress syndrome

Note: WBC = white blood cell count.

Patient management

The scope of the initial assessment is to establish the grade and the severity of OHSS. In the first instance, telephone assessment [48] may represent a useful modality to establish the presence and the grade of OHSS. It is important to understand if there are specific conditions referable to OHSS in the recent history of the patient and if abdominal pain, shortness of breath or impression of reduced urine output have occurred. These features can indicate severe OHSS with the eventual presence of specific respiratory, renal, or ovarian impairment which requires hospitalization [57–61]. On the other hand, it has to be mentioned that face-to-face clinical assessment allows examination and investigations with the aim of clarifying the diagnosis and severity of the patient's conditions. Vigilance about the severity of OHSS signs should be recommended to both clinicians and patients. The severity of OHSS is worsening if any of the signs reported in Table 69.5 occurs.

With regard to mild stages of OHSS, they may be managed on an outpatient basis. No specific evidence is existent regarding fluid intake; in view of this, it should be recommended to thirst with at least 1 litre/day [51]. Fluid input–output charts could be registered by the patients their self. Urine output of less than 1000 mL per 24 hours or a positive fluid balance of greater than 1000 mL over 24 hours should prompt medical review to assess severity. Considering pain relief therapy, nonsteroidal anti-inflammatory drugs (NSAIDs) should be avoided as they may impair renal function in OHSS patients [51], whereas paracetamol and oral opiates including codeine can be safely administered. Women with severe OHSS are at increased risk of thromboembolism [62, 63]; in this view, although there are no trials on this argument, thromboprophylaxis should be provided for these women [64]. Finally, it has to be mentioned that ultrasound guided paracentesis may represent a safe alternative to hospitalization in patients with severe OHSS [65, 66].

Criteria for patients' admission to the hospital are not categorically defined. Considering this, hospitalization should be evaluated with reference to the clinical features, social factors, and the available expertise [48]. However, conditions for which hospitalization may be considered are reported in Table 69.6. In patients with severe OHSS who have persisting dehydration and haemoconcentration despite an adequate fluid replacement, invasive haemodynamic monitoring may be needed with input from anaesthetic/intensive care colleagues. Intensive care may also be required for women with critical OHSS showing specific complications such as thromboembolism, acute respiratory distress syndrome (ARDS), and renal failure. In this view, assisted reproduction centres should always be in contact with emergency units, providing an adequate expertise available for the care of women admitted with OHSS.

TABLE 69.5 Signs of OHSS Increased Severity

Increasing abdominal distension and pain
Shortness of breath
Tachycardia or hypotension
Reduced urine output (less than 1000 mL/24 hours) or positive fluid balance (more than 1000 mL/24 hours)
Weight gain and increased abdominal girth
Increasing haematocrit (>45%)

TABLE 69.6 Conditions Requiring Patient Admission to the Hospital

No satisfactory pain control
No adequate fluid intake due to nausea
Signs of worsening OHSS despite outpatient intervention
Inability to attend for regular outpatient follow-up
Critical OHSS

Monitoring of women with OHSS should have the scope to intercept changes in the severity of the condition and complications at an early stage. Daily check-up of body weight, abdominal girth, fluid intake, and fluid output should be performed, along with blood samples reporting full blood count, haematocrit, serum electrolytes, osmolality, and liver function tests. Depending on the clinical features, arterial blood gases, ECG, chest X-ray, and other imaging may be necessary. Signs of worsening OHSS include increasing abdominal girth, weight gain, oliguria with positive fluid balance, and elevated haematocrit. Conversely, recovery is undelighted by reduction in abdominal girth and body weight as well as normalization of diuresis and haematocrit [67, 68]. On the other hand, it seems that C-reactive protein levels correlate with other markers of OHSS such as abdominal girth and weight and may have a role in monitoring severity [67, 69]. Physiological fluid replacement by the oral route, guided by thirst, represents the first approach to correct dehydration [48]. Moreover, it seems that excessive intravenous fluid therapy with crystalloids may be dangerous as it may potentially worsening the ascites in the presence of increased capillary permeability. However, when persisting haemoconcentration and acute dehydration, initial correction with crystalloids and/or colloids may be useful for those women who are not able to maintain adequate oral intake. Human albumin solution 25% may be used as a plasma volume expander in doses of 50–100 g, infused over four hours and repeated 4 to 12-hourly [51]. Six per cent hydroxyethyl starch solution (HES) has been compared to human albumin as colloid solutions for treatment of severe OHSS in 16 patients [70]. It seems that patients who received HES had shorter duration of hospital stay (15.7 ± 5.7 vs 19.0 ± 8.2 days) and higher urine output than women treated with albumin. Moreover, fewer abdominal paracenteses and pleural thoracenteses (33% vs 80%) were needed compared to patients who received albumin. No difference in adverse effects was reported. These results underlined that 6% HES may be superior to albumin as a colloid solution for the treatment of severe OHSS. However, the small sample size of the cohort and the study design, are not so robust to draw definitive conclusions. Strict fluid balance recording should be followed for these patients with accurate urine output measurement and invasive haemodynamic monitoring when persisting haemoconcentration or low urine output. Oliguria despite adequate fluid replacement may in some cases respond to paracentesis [71], however dopamine infusion or oral doxapamine administration is also described in literature to treat severe OHSS [57–59]. With regard to diuretics use for management of fluid balance in women with OHSS, it seems that there is a risk of worsening hypovolemia if diuretics are administered without correcting dehydration. However, careful use of diuretics should be recommended in women who maintain the condition of oliguria despite an adequate fluid replacement, especially if

any tense ascites that may have been contributing to oliguria has been drained by paracentesis [57, 71, 72]. Severe hypovolemia may rarely lead to life-threatening risk for arterial or venous thromboembolism, hence, prophylactic anticoagulation is warranted for patients with severe OHSS from the time of diagnosis through the first trimester of pregnancy [25, 67, 73].

Prevention of OHSS

Primary prevention: The use of a GnRH antagonist protocol

Several studies described that the use of gonadotropin-releasing hormone (GnRH) antagonists protocols results in a lower incidence of OHSS compared with protocols that use a GnRH agonist [63]. Indeed, it seems that the mechanism of reduction in circulating oestradiol levels seen with GnRH antagonist suppression would be in favour to the lower risk of OHSS. Given this, GnRH antagonist protocol should be recommended for PCOS/polycystic ovary morphology (PCOM) patients and for those who are predicted high responders [33, 74–77]. In addition, the use of a GnRH antagonist for ovarian suppression and lowering of OHSS rate, have been also described by several systematic reviews [49]. A Cochrane review analysing data from 29 randomized controlled trials (RCTs) showed a statistically significant lower incidence of OHSS in the GnRH antagonist group (odds ratio [OR] 0.43, 95% CI 0.33–0.57) and no difference in live birth rates compared to women who underwent GnRH agonist protocol [78–82]. With regard to the addition of clomiphene to controlled ovarian stimulation in antagonist protocols, several studies described a reduction of OHSS risk. However, the heterogeneity of the population included (i.e. patients who underwent minimal stimulation protocols) led to a difficult interpretation of the results [82].

Secondary prevention: Agonist trigger for final oocyte maturation prior to retrieval

GnRH agonist trigger has been widely compared with hCG trigger for final oocyte maturation to investigate whether the OHSS incidence may be reduced. Several RCTs provided strong evidence that the administration of a GnRH agonist trigger is associated with a significant decrease in the development of OHSS, both in donors/women with PCOS [83–86]. Bodry et al. evaluated a cohort of oocyte donors over 4052 stimulation cycles in which hCG or GnRH agonist was administered on physician discretion [87–94]. In accordance with other reports, moderate/severe OHSS occurs less frequently in those women receiving GnRH agonist trigger compared with hCG (0% [0/1519] vs 0.87% [22/2533], respectively) [91]. However, for the lower incidence of OHSS in fresh autologous cycles reported in a Cochrane review published in 2014 summarizing the results of 17 RCTs [91], the authors also reported a lower live-birth rate (OR 0.47, 95% CI 0.31–0.70; five RCTs, 532 women, moderate-quality evidence) in fresh autologous cycles [95]. The mechanism by which live birth rate is impaired, is probably based on the rapid decrement and dramatic post-luteal drop in LH support compared to hCG for maturation, this results in luteal phase insufficiency. Along this line, the strategy of co-administration of GnRH agonist trigger with low dose hCG (1000 IU, 500 IU, or 250 IU every third day after retrieval) for luteal support may restore pregnancy rates [95] and still reduce OHSS with a trend towards higher incidence of moderate OHSS with the 1000 IU dosing compared to the lower doses [96]. Moreover, an RCT of 384 patients showed that GnRH agonist trigger in association with a single bolus of

1500 IU of hCG after the oocyte retrieval decreased OHSS in high-risk patients with an increased risk of moderate-to-late onset of OHSS when patients received a second bolus of 1500 IU (one the day of retrieval and one the subsequent day) [89, 97].

Tertiary prevention: Co-adjuvant treatments

Aspirin or metformin

The use of aspirin for OHSS prevention has been investigated by two randomized trials [98, 99]. These studies found a lower incidence of OHSS in patients treated with a daily dose of 100 mg aspirin from the first day of stimulation until the day of the pregnancy test/detection of embryonic cardiac activity. Indeed, mechanism at the base of OHSS consists in a releasing of substances (histamine, serotonin, platelet-derived growth factor, lysophosphatidic acid) due to an activation of platelet by VEGF. Given this, aspirin has been suggested to have a potential role in reduction of OHSS risk [98, 99]. With regard to the metformin, its action of improvement of intraovarian hyperandrogenism contributes to the reduction of oestradiol secretion due to the decrement of the number of periovulatory follicles. Several studies have shown that metformin at the onset of downregulation during ovarian stimulation until oocyte retrieval in PCOS women reduces the risk of OHSS [100]. In accordance, several RCTs and a systematic review have supported this conclusion [100, 101]. However, this effect has not yet been demonstrated in patients with PCOM only [101–105] and those who are of standard weight [106].

Vasopressin-induced vascular endothelial growth factor secretion blockade

Relcovaptan is a non-peptide vasopressin receptor antagonist able to contrast the VEGF action by adjusting vascular smooth muscle proliferation and vasoconstriction. A study on hyperstimulated rat models, treated with relcovaptan showed lower concentrations of the VEGF-A in the peritoneal fluid, lower weight gain, and decreased number of corpora lutea [107].

Withholding gonadotropins

Several studies investigated the practice of withholding gonadotropins at the end of controlled ovarian stimulation for up to four days to decrease OHSS risk. It seems that coasting is associated with a lower risk of OHSS without compromising the pregnancy rate [108]. However, this evidence is not supported by a systematic review of four RCTs which described that the risk of OHSS is not decreased with coasting [109–112]. On the other hand, one study concluded the incidence of OHSS is higher when withholding gonadotropins is applied to the controlled ovarian stimulation [113]. In addition, the optimal length of coasting has not yet been established, with poor cohort studies suggesting that coasting four or more days reduces implantation rates [114].

Dopamine-receptor agonist treatment

Treatment with dopamine-receptor agonist has been supposed to result in a decreasing VEGF production with consequent reduction of OHSS symptoms and grade. Several randomized controlled studies investigated the administration of dopamine agonist to reduce the severity of OHSS with success of treatment achieved especially for patients with moderate OHSS [115–120]. In addition, several systematic reviews compared the administration of cabergoline with placebo. A review of seven studies in 858 women found that the incidence of OHSS was significantly lower in women treated with cabergoline compared with no treatment (RR 0.38, CI 0.29–0.51, P < .00001), without any impact reported on pregnancy rates (RR 1.02, 95% CI 0.78–1.34, four studies, 561

women) [116]. In this context, a recent Cochrane review of 22 RCTs concluded that dopamine agonists are effective in reducing the incidence of moderate or severe OHSS in patients at high risk for OHSS when compared with placebo or no treatment [121]. With regard to dosage and timing of administration, most of the studies suggested the use of cabergoline 0.5 mg orally daily for seven or eight days starting on the day of oocyte pickup or hCG trigger [115, 116, 118, 119]. On the other hand, other studies gave oral cabergoline 0.5 mg per day for three weeks beginning on the day after the oocyte retrieval [117]. Finally, 0.25 mg of cabergoline daily for eight days from the day of HCG administration has been also proposed [120]. A recent retrospective study including patients who underwent GnRH antagonist cycles with GnRH agonist trigger suggested that the administration of cabergoline should be considered at the time of triggering in women at risk of OHSS. However, the authors concluded that larger, prospective studies in groups receiving an hCG trigger are warranted to support these findings [122]. In addition, it has to be mentioned that data about pregnancy outcomes were scarcely reported and, although it seems that dopamine agonists might improve pregnancy outcomes, the presence of mild side effects such as stomach upsets, feeling sick, or dizziness must be taken into account [123].

Albumin and calcium administration

The rationale of using albumin as method to prevent of OHSS relies on the fact that it has low molecular weight as well as an average half-life of 20 days, acting as an increaser of plasma oncotic pressure and contrasting the permeability effect of angiotensin II. Moreover, it binds vasoactive substances, such as factors related to the renin-angiotensin system and VEGF. However, contrasting data exist about the role of albumin in the prevention of OHSS. Older studies demonstrated a trend towards the positive effect of human albumin administrated at the time of oocyte retrieval, reducing the incidence of moderate-to-severe OHSS compared with no treatment [121]. However, recent evidence failed to replicate the same findings [124–126]. In addition, two systematic reviews concluded that albumin is not effective in preventing OHSS [115, 116, 127, 128] also reporting a significant lowering of pregnancy rate in patients who received albumin around oocyte retrieval compared with no treatment (RR 0.8, 95% CI 0.57–1.12) [129, 130]. On the other hand, similar results have been reported when comparing albumin with other methods such as use of HES [129] or cooating [131]. It is also important to remember that the administration of albumin (as blood-derived product) may cause allergic reactions, anaphylactic reactions as well as rare but possible transmission of viral or unidentified diseases. Similarly, the use of IV calcium (10 mL of 10% calcium gluconate in 200 mL normal saline) around the day of oocyte retrieval and thereafter has been investigated as a strategy to reduce OHSS. Calcium is described to inhibit the secretion renin mediated by cAMP resulting in a reduction of angiotensin II and subsequent decrease of VEGF production. A RCT compared the use IV calcium and normal saline in 200 women at risk for OHSS reporting higher incidence of moderate and severe OHSS in women treated with normal saline [117], without impact on clinical pregnancy or ongoing pregnancy rate between the groups. In addition, evidence suggests that IV calcium is as effective as cabergoline in lowering the OHSS risk in PCOS women [132] and in its prevention [133, 134].

Cryopreservation

Cryopreservation of all embryos also noted as “freeze-all” strategy is a safe approach to avoid the endogenous hCG rise in fresh

transfer cycles, which may be responsible for late-onset OHSS symptoms and its longer duration. A small RCT reported the successful use of elective cryopreservation as method to prevent OHSS, compared to albumin in preventing mild, moderate, and severe OHSS in high-risk women [135]. In another RCT including 125 patients treated with the cryopreservation strategy a lower incidence of OHSS was reported with respect to controls who underwent fresh embryo transfers [136]. However, a systematic review, including only these two studies concluded that there was not robust evidence to support cryopreservation as method to reduce OHSS risk [137]. On the other hand, it has been reported that also in “freeze-all” cycles with agonist trigger, there might persist a residual incidence of severe OHSS [138, 139]. Indeed, “freeze-all” strategy may have encouraged the use of more aggressive ovarian stimulation with consequent higher risk for OHSS development.

In vitro maturation (IVM) of immature oocytes

IVM can be considered as an alternative method for fertility treatment in hyper-responding patients who can be at high risk for OHSS (e.g. PCOS/PCOM patients) [140, 141].

Focus on letrozole

High serum oestrogen levels have been correlated with elevated risk of OHSS [142]. In this contest, letrozole as nonsteroidal aromatase inhibitor, acts impeding the conversion of androgens into oestrogens induced by the aromatase [143]. Recent evidence showed that the use of letrozole after oocyte retrieval may reduce oestrogen concentrations with consequent possibility to decrease the OHSS incidence [144, 145]. Nevertheless, although during the last few years several clinical trials described the efficacy of letrozole in reducing the incidence of OHSS, other studies failed to report the same findings showing that letrozole only reduce the oestrogen levels without prevention of occurrence of the syndrome [146, 147]. The effect of different doses of letrozole on the incidence of OHSS after oocyte retrieval during IVF in patients at high-risk for OHSS was recently investigated. Results showed that 2.5 mg, 5.0 mg, and 7.5 mg daily for five days are able to decrease the oestrogen levels and VEGF. However, although the doses of 2.5 mg and 5 mg showed a slightly decrease of OHSS incidence, the higher dosage of 7.5 mg determined a significant reduction, indicating its effectiveness in limiting OHSS [147]. On the other hand, letrozole was also compared with other compounds such as aspirin for prevention of early OHSS, with satisfactory results in favour of letrozole which was more effective than aspirin in decreasing the incidence of moderate and severe early-onset OHSS. In this study, the authors indicated that OHSS might be caused by a luteolytic effect rather modulation of VEGF [146]. Another study, by Wang et al. showed that 5 mg of letrozole administrated during luteal phase can significantly decrease serum oestrogen levels on the second, fifth, and eighth days after oocyte retrieval compared with the control group but were not effective in reducing the incidence of severe OHSS [148]. These results were confirmed by the same team two years later [149]. To date, the guideline for “Prevention and Treatment of moderate and severe ovarian hyperstimulation syndrome” do not mention Letrozole as agent for OHSS prevention [48]. However, a recent systematic review and meta-analysis interestingly showed that Letrozole could decrease the incidence of total OHSS and moderate + severe OHSS in high-risk women, while it seems to be not effective on the prevention of mild, moderate, and severe OHSS, individually [150]. This is in line with results reported by Wang et al. [148, 149].

Focus on luteal phase support

Luteal phase support (LPS) represents a crucial step in IVF cycles followed by fresh embryo transfer, with a wealth of studies investigating its efficiency, route, and duration. Although different routes, alone or in combination, have been proposed during the last decade, the vaginal route seems to be the preferential route for LPS [151, 152]. On the other hand, there is a tendency to abandon the use of hCG as agent for luteal phase supplementation. In this context, the results of a Cochrane meta-analysis showed that hCG is not superior to progesterone for LPS; moreover, analysis of pooled data pointed toward a higher risk of OHSS when hCG was administered in the luteal phase [153–155]. Considering this, the use of hCG for LPS should be avoided especially for those women at high risk for OHSS. Conversely, the option of LPS with sole GnRH agonist have been proposed as a possible approach for LPS in patients having elevated risk for OHSS [156]. Although promising, this option was not able to eliminate the risk for severe OHSS [157, 158].

Conclusions

OHSS is a well-described complication of controlled ovarian stimulation. The goal of the clinical practice should be to identify patients at high risk for this condition prior to starting ovarian stimulation. Accurate evaluation of the woman's endocrine profile should be carefully conducted in order to select a tailored protocol for each patient with the aim to minimize the risk for OHSS. To date, the use of GnRH antagonist protocol with a GnRH agonist (with or without low dose hCG) to trigger final oocyte maturation seems to represent an effective strategy to prevent OHSS. In addition, other expedients such as the use of cabergoline and cryopreservation of all embryos might be of great benefit. In case of failure of the strategies for OHSS prevention, with patients experiencing severe OHSS, fluid resuscitation, supportive care, paracentesis, and prophylactic anticoagulation are warranted.

References

- Santos-Ribeiro S, Polyzos NP, Stouffs K, De Vos M, Seneca S, Tournaye H, et al. Ovarian hyperstimulation syndrome after gonadotropin-releasing hormone agonist triggering and "freeze-all": In-depth analysis of genetic predisposition. *J Assist Reprod Genet.* 2015 Jul;32(7):1063–8.
- Humaidan P, Kol S, Papanikolaou EG. GnRH agonist for triggering of final oocyte maturation: Time for a change of practice? *Hum Reprod Update.* 2011 Jul;17(4):510–24.
- Steward RG, Lan L, Shah AA, Yeh JS, Price TM, Goldfarb JM, et al. Oocyte number as a predictor for ovarian hyperstimulation syndrome and live birth: An analysis of 256,381 in vitro fertilization cycles. *Fertil Steril.* 2014;101(4):967–73.
- Luke B, Brown MB, Morbeck DE, Hudson SB, Coddington CC, Stern JE. Factors associated with ovarian hyperstimulation syndrome (OHSS) and its effect on assisted reproductive technology (ART) treatment and outcome. *Fertil Steril.* 2010 Sep;94(4):1399–404.
- Jayaprakasan K, Chan Y, Islam R, Haoula Z, Hopkisson J, Coomarasamy A, et al. Prediction of in vitro fertilization outcome at different antral follicle count thresholds in a prospective cohort of 1,012 women. *Fertil Steril.* 2012 Sep;98(3):657–63.
- Papanikolaou EG, Pozzobon C, Kolibianakis EM, Camus M, Tournaye H, Fatemi HM, et al. Incidence and prediction of ovarian hyperstimulation syndrome in women undergoing gonadotropin-releasing hormone antagonist in vitro fertilization cycles. *Fertil Steril.* 2006 Jan;85(1):112–20.
- Kahnberg A, Enskog A, Brännström M, Lundin K, Bergh C. Prediction of ovarian hyperstimulation syndrome in women undergoing in vitro fertilization. *Acta Obstet Gynecol Scand.* 2009 Dec 26;88(12):1373–81.
- Goldsman MP, Pedram A, Dominguez CE, Ciuffardi I, Levin E, Asch RH. Increased capillary permeability induced by human follicular fluid: A hypothesis for an ovarian origin of the hyperstimulation syndrome. *Fertil Steril.* 1995;63(2):268–72.
- Bergh PA, Navot D. Ovarian hyperstimulation syndrome: A review of pathophysiology. *J Assist Reprod Genet.* 1992;9:429–38.
- Geva E, Jaffe RB. Role of vascular endothelial growth factor in ovarian physiology and pathology. *Fertil Steril.* 2000;74:429–38.
- Levin ER, Rosen GF, Cassidenti DL, Yee B, Meldrum D, Wisot A, et al. Role of vascular endothelial cell growth factor in ovarian hyperstimulation syndrome. *J Clin Invest.* 1998 Dec 1;102(11):1978–85.
- Neulen J, Yan Z, Raczek S, Weindel K, Keck C, Weich HA, et al. Human chorionic gonadotropin-dependent expression of vascular endothelial growth factor/vascular permeability factor in human granulosa cells: Importance in ovarian hyperstimulation syndrome. *J Clin Endocrinol Metab.* 1995;80(6):1967–71.
- McClure N, Healy DL, Rogers PAW, Sullivan J, Robertson DM, Haning RV, et al. Vascular endothelial growth factor as capillary permeability agent in ovarian hyperstimulation syndrome. *Lancet.* 1994 Jul 23;344(8917):235–6.
- Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohí J, Simón C. The pathogenesis of ovarian hyperstimulation syndrome: In vivo studies investigating the role of interleukin-1 β , interleukin-6, and vascular endothelial growth factor. *Fertil Steril.* 1999 Mar;71(3):482–9.
- Friedlander MA, De Mola JRL, Goldfarb JM. Elevated levels of interleukin-6 in ascites and serum from women with ovarian hyperstimulation syndrome. *Fertil Steril.* 1993;60(5):826–33.
- Whelan JG, Vlahos NF. The ovarian hyperstimulation syndrome. *Fertil Steril.* 2000;73(5):883–96.
- Delbaere A, De Maertelaer V, Bergmann PJM, Staroukine M, Gervy-Decoster C, Camus M, et al. Increased angiotensin II in ascites during severe ovarian hyperstimulation syndrome: Role of early pregnancy and ovarian gonadotropin stimulation. *Fertil Steril.* 1997;67(6):1038–45.
- Morris RS, Wong IL, Kirkman E, Gentschein E, Paulson RJ. Endocrinology: Inhibition of ovarian-derived prorenin to angiotensin cascade in the treatment of ovarian hyperstimulation syndrome. *Hum Reprod.* 1995 Jun;10(6):1355–8.
- Ashrafi M, Bahmanabadi A, Akhond MR, Arabipoor A. Predictive factors of early moderate/severe ovarian hyperstimulation syndrome in non-polycystic ovarian syndrome patients: A statistical model. *Arch Gynecol Obstet.* 2015 Nov 1;292(5):1145–52.
- Johnson MD, Williams SL, Seager CK, Liu JH, Barker NM, Hurd WW. Relationship between human chorionic gonadotropin serum levels and the risk of ovarian hyperstimulation syndrome. *Gynecol Endocrinol.* 2014;30(4):294–7.
- Sousa M, Cunha M, Teixeira da Silva J, Oliveira C, Silva J, Viana P, et al. Ovarian hyperstimulation syndrome: A clinical report on 4894 consecutive ART treatment cycles. *Reprod Biol Endocrinol.* 2015 Jun 23;13(1).
- Mathur RS, Akande AV, Keay SD, Hunt LP, Jenkins JM. Distinction between early and late ovarian hyperstimulation syndrome. *Fertil Steril.* 2000;73(5):901–7.
- Aramwit P, Pruksananonda K, Kasettrat N, Jammeechai K. Risk factors for ovarian hyperstimulation syndrome in Thai patients using gonadotropins for in vitro fertilization. *Am J Heal Pharm.* 2008 Jun 15;65(12):1148–53.
- Danninger B, Brunner M, Obruc A, Feichtinger W. Prediction of ovarian hyperstimulation syndrome of baseline ovarian volume prior to stimulation. *Hum Reprod.* 1996;11(8):1597–9.

25. Delvigne A, Demoulin A, Smitz J, Donnez J, Koninckx P, Dhont M, et al. Endocrinology: The ovarian hyperstimulation syndrome in in-vitro fertilization: A belgian multicentric study. I. clinical and biological features. *Hum Reprod.* 1993;8(9):1353–60.
26. Lee TH, Liu CH, Huang CC, Wu YL, Shih YT, Ho HN, et al. Serum anti-müllerian hormone and estradiol levels as predictors of ovarian hyperstimulation syndrome in assisted reproduction technology cycles. *Hum Reprod.* 2008;23(1):160–7.
27. Swanton A, Storey L, McVeigh E, Child T. IVF outcome in women with PCOS, PCO and normal ovarian morphology. *Eur J Obstet Gynecol Reprod Biol.* 2010;149(1):68–71.
28. Nakhuda GS, Chu MC, Wang JG, Sauer MV, Lobo RA. Elevated serum müllerian-inhibiting substance may be a marker for ovarian hyperstimulation syndrome in normal women undergoing in vitro fertilization. *Fertil Steril.* 2006 May;85(5):1541–3.
29. Tal R, Seifer DB, Khanimov M, Malter HE, Grazi RV, Leader B. Characterization of women with elevated antimüllerian hormone levels (AMH): Correlation of AMH with polycystic ovarian syndrome phenotypes and assisted reproductive technology outcomes. *Am J Obstet Gynecol.* 2014;211(1):59.e1–59.e8.
30. Ocal P, Sahmay S, Cetin M, Izre T, Gurlalp O, Cepni I. Serum anti-müllerian hormone and antral follicle count as predictive markers of OHSS in ART cycles. *J Assist Reprod Genet.* 2011 Dec;28(12):1197–203.
31. Moos J, Rezabek K, Filova V, Moosova M, Pavelkova J, Peknicova J. Comparison of follicular fluid and serum levels of inhibin A and inhibin B with calculated indices used as predictive markers of ovarian hyperstimulation syndrome in IVF patients. *Reprod Biol Endocrinol.* 2009 Aug 24;7:86.
32. Jayaprakasan K, Herbert M, Moody E, Stewart JA, Murdoch AP. Estimating the risks of ovarian hyperstimulation syndrome (OHSS): Implications for egg donation for research. *Hum Fertil.* 2007 Sep;10(3):183–7.
33. Åbyholm T, Barlow D, Devroey P, Diedrich K, Donnez J, Düring V, et al. Treatment with the gonadotrophin-releasing hormone antagonist ganirelix in women undergoing ovarian stimulation with recombinant follicle stimulating hormone is effective, safe and convenient: Results of a controlled, randomized, multicentre trial. *Hum Reprod.* 2000;15(7):1490–8.
34. Mathur RS, Tan BK. British fertility society policy and practice committee: Prevention of ovarian hyperstimulation syndrome. *Hum Fertil.* 2014 Dec 1;17(4):257–68.
35. Tarlatzi TB, Venetis CA, Devreker F, Englert Y, Delbaere A. What is the best predictor of severe ovarian hyperstimulation syndrome in IVF? A cohort study. *J Assist Reprod Genet.* 2017 Oct 1;34(10):1341–51.
36. Reljić M, Vlaisavljević V, Gavrić V, Kovačić B. Number of oocytes retrieved and resulting pregnancy: Risk factors for ovarian hyperstimulation syndrome. *J Reprod Med Obstet Gynecol.* 1999 Aug;44(8):713–8.
37. D'Angelo A, Davies R, Salah E, Nix BA, Amso NN. Value of the serum estradiol level for preventing ovarian hyperstimulation syndrome: A retrospective case control study. *Fertil Steril.* 2004;81(2):332–6.
38. Hendriks DJ, Klinkert ER, Bancsi LFJMM, Looman CWN, Habbema JDF, Te Velde ER, et al. Use of stimulated serum estradiol measurements for the prediction of hyperresponse to ovarian stimulation in in vitro fertilization (IVF). *J Assist Reprod Genet.* 2004 Mar;21(3):65–72.
39. Al-Shawaf T, Zosmer A, Hussain S, Tozer A, Panay N, Wilson C, et al. Prevention of severe ovarian hyperstimulation syndrome in IVF with or without ICSI and embryo transfer: A modified “coasting” strategy based on ultrasound for identification of high-risk patients. *Hum Reprod.* 2001;16(1):24–30.
40. Grochowski D, Wolczyński S, Kuczyński W, Domitrz J, Szamatowicz J, Szamatowicz M. Correctly timed coasting reduces the risk of ovarian hyperstimulation syndrome and gives good cycle outcome in an in vitro fertilization program. *Gynecol Endocrinol.* 2001;15(3):234–8.
41. Kawaguchi R, Ueda S, Tsuji Y, Haruta S, Kanayama S, Yamada Y, et al. Massive ovarian edema in pregnancy after ovulation induction using clomiphene citrate. *Arch Gynecol Obstet.* 2008 Apr;277(4):375–8.
42. Mitchell SY, Fletcher HM, Williams E. Ovarian hyperstimulation syndrome associated with clomiphene citrate. *West Indian Med J.* 2001;50(3):227–9.
43. Gil Navarro N, Garcia Grau E, Pina Pérez S, Ribot Luna L. Ovarian torsion and spontaneous ovarian hyperstimulation syndrome in a twin pregnancy: A case report. *Int J Surg Case Rep.* 2017;34:66–8.
44. Sridev S, Barathan S. Case report on spontaneous ovarian hyperstimulation syndrome following natural conception associated with primary hypothyroidism. *J Hum Reprod Sci.* 2013 Apr;6(2):158–61.
45. Cardoso CG, Graça LM, Dias T, Clode N, Soares L. Spontaneous ovarian hyperstimulation and primary hypothyroidism with a naturally conceived pregnancy. *Obstet Gynecol.* 1999 May;93 (5 SUPPL.):809–11.
46. Panagiotopoulou N, Byers H, Newman WG, Bhatia K. Spontaneous ovarian hyperstimulation syndrome: Case report, pathophysiological classification and diagnostic algorithm. *Eur J Obstet Gynecol Reprod Biol.* 2013;169:143–8.
47. Lussiana C, Guani B, Restagno G, Rovei V, Menato G, Revelli A, et al. Ovarian hyper-stimulation syndrome after spontaneous conception. *Gynecol Endocrinol.* 2009;25(7):455–9.
48. Pfeifer S, Butts S, Dumesic D, Fossum G, Gracia C, La Barbera A, et al. Prevention and treatment of moderate and severe ovarian hyperstimulation syndrome: A guideline. *Fertil Steril.* 2016 Dec 1; 106(7):1634–47.
49. Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, Kolibianakis E, et al. ESHRE guideline: Ovarian stimulation for IVF/ICSI. *Hum Reprod Open.* 2020 Feb 1;2020(2):1–13.
50. Papanikolaou EG, Tournaye H, Verpoest W, Camus M, Vernaevé V, Van Steirteghem A, et al. Early and late ovarian hyperstimulation syndrome: Early pregnancy outcome and profile. *Hum Reprod.* 2005;20(3):636–41.
51. Evbuomwan I. The role of osmoregulation in the pathophysiology and management of severe ovarian hyperstimulation syndrome. *Hum Fertil.* 2013 Sep;16(3):162–7.
52. Memarzadeh MT. A fatal case of ovarian hyperstimulation syndrome with perforated duodenal ulcer. *Hum Reprod.* 2010; 25:808–9.
53. Golan A, Ron-El R, Herman A, Soffer Y, Weinraub Z, Caspi E. Ovarian hyperstimulation syndrome: An update review. *Obstet Gynecol Surv.* 1989;44(6):430–40.
54. Golan A, Weissman A. Symposium: Update on prediction and management of OHSS: A modern classification of OHSS. *Reprod Biomed Online.* 2009;19(1):28–32.
55. Navot D, Bergh PA, Laufer N. Ovarian hyperstimulation syndrome in novel reproductive technologies: Prevention and treatment. *Fertil Steril.* 1992;58:249–61.
56. Rizk B, Aboulghar MA. Classification, pathophysiology and management of ovarian hyperstimulation syndrome. In: *In-Vitro Fertilization and Assisted Reproduction*, Brinsden P (ed.). New York/London: The Parthenon Publishing Group, pp. 131–55, 1999.
57. Ferraretti AP, Gianaroli L, Diotallevi L, Festi C, Trounson A. Dopamine treatment for severe ovarian hyperstimulation syndrome. *Hum Reprod.* 1992;7(2):180–3.
58. Lin J, Li X, Zhang L. Long hospitalization for severe ovarian hyperstimulation syndrome with persistent right hydrothorax and two occurrences of pneumonia. *Int J Gynecol Obstet.* 2017 Mar 1; 136(3):350–2.
59. Abramov Y, Elchalal U, Schenker JG. Pulmonary manifestations of severe ovarian hyperstimulation syndrome: A multicenter study. *Fertil Steril.* 1999 Apr;71(4):645–51.
60. Abramov Y, Elchalal U, Schenker JG. An “epidemic” of severe OHSS: A price we have to pay? *Hum Reprod.* 1999;14:2181–3.

61. Omari A, Ghazal-Aswad W, Sidky S, Bassam A MK. Ovarian salvage in bilaterally complicated severe ovarian hyperstimulation syndrome. *Fertil Steril.* 2011 Aug;96(2).
62. Danolić D, Kasum M, Puljiz M, Alvir I, Tomica D, Mamić I, et al. The risk of hypercoagulability in ovarian hyperstimulation syndrome. *Acta Clin Croat.* 2015;54(2):186–91.
63. Magnusson Å, Källen K, Thurin-Kjellberg A, Bergh C. The number of oocytes retrieved during IVF: A balance between efficacy and safety. *Hum Reprod.* 2018 Jan 1;33(1):58–64.
64. Lamont MC, McDermott C, Thomson AJ, Greer IA. United Kingdom recommendations for obstetric venous thromboembolism prophylaxis: Evidence and rationale. *Semin Perinatol.* 2019;43:222–8.
65. Lincoln SR, Opsahl MS, Blauer KL, Black SH, Schulman JD. Aggressive outpatient treatment of ovarian hyperstimulation syndrome with ascites using transvaginal culdocentesis and intravenous albumin minimizes hospitalization. *J Assist Reprod Genet.* 2002;19(4):159–63.
66. Qublan HS, Al-Taani MI, Megdadi MF, Metri RM, Al-Ahmad N. Multiple transvaginal ascitic fluid aspirations improves the clinical and reproductive outcome in patients undergoing in vitro fertilisation treatment complicated by severe early ovarian hyperstimulation syndrome. *J Obstet Gynaecol.* 2012 May;32(4):379–82.
67. Practice Committee of American Society for Reproductive Medicine. Ovarian hyperstimulation syndrome. *Fertil Steril.* 2008 Nov;90(5 Suppl):S188–93.
68. Fábregues F, Balasch J, Manau D, Jiménez W, Arroyo V, Creus M, et al. Haematocrit, leukocyte and platelet counts and the severity of the ovarian hyperstimulation syndrome. *Hum Reprod.* 1998;13(9):2406–10.
69. Nowicka MA, Fritz-Rdzanek A, Grzybowski W, Walecka I, Niemiec KT, Jakimiuk AJ. C-reactive protein as the indicator of severity in ovarian hyperstimulation syndrome. *Gynecol Endocrinol.* 2010 Jun;26(6):399–403.
70. Abramov Y, Fatum M, Abrahamov D, Schenker JG. Hydroxyethyl-starch versus human albumin for the treatment of severe ovarian hyperstimulation syndrome: A preliminary report. *Fertil Steril.* 2001;75(6):1228–30.
71. Maslovitz S, Jaffa A, Eytan O, Wolman I, Many A, Lessing JB, et al. Renal blood flow alteration after paracentesis in women with ovarian hyperstimulation. *Obstet Gynecol.* 2004 Aug;104(2):321–6.
72. Tsunoda T, Shibahara H, Hirano Y, Suzuki T, Fujiwara H, Takamizawa S, et al. Treatment for ovarian hyperstimulation syndrome using an oral dopamine prodrug, docarpamine. *Gynecol Endocrinol.* 2003;17(4):281–6.
73. Rova K, Passmark H, Lindqvist PG. Venous thromboembolism in relation to in vitro fertilization: An approach to determining the incidence and increase in risk in successful cycles. *Fertil Steril.* 2012 Jan;97(1):95–100.
74. Ludwig M, Felberbaum RE, Devroey P, Albano C, Riethmüller-Winzen H, Schüler A, et al. Significant reduction of the incidence of ovarian hyperstimulation syndrome (OHSS) by using the LHRH antagonist Cetrorelix (Cetrotide®) in controlled ovarian stimulation for assisted reproduction. *Arch Gynecol Obstet.* 2000;264(1):29–32.
75. Qiao J, Zhang Y, Liang X, Ho T, Huang H-Y, Kim S-H, et al. A randomised controlled trial to clinically validate follitropin delta in its individualised dosing regimen for ovarian stimulation in Asian IVF/ICSI patients. *Hum Reprod.* 2021 Aug 18;36(9):2452–62.
76. Borges E, Braga DPAF, Setti AS, Vingris LS, Figueira RCS, Iaconelli A. Strategies for the management of OHSS: Results from freezing-all cycles. *J Bras Reprod Assist.* 2016;20(1):8–12.
77. Firouzabadi RD, Ahmadi S, Oskouian H, Davar R. Comparing GnRH agonist long protocol and GnRH antagonist protocol in outcome the first cycle of ART. *Arch Gynecol Obstet.* 2010 Jan;281(1):81–5.
78. Ludwig M, Katalinic A, Diedrich K. Use of GnRH antagonists in ovarian stimulation for assisted reproductive technologies compared to the long protocol: Meta-analysis. *Arch Gynecol Obstet.* 2001;265(4):175–82.
79. Mancini F, Tur R, Martinez F, Coroleu B, Rodríguez I, Barri PN. Gonadotrophin-releasing hormone-antagonists vs long agonist in in-vitro fertilization patients with polycystic ovary syndrome: A meta-analysis. *Gynecol Endocrinol.* 2011 Mar;27(3):150–5.
80. Xiao J, Chen S, Zhang C, Chang S. Effectiveness of GnRH antagonist in the treatment of patients with polycystic ovary syndrome undergoing IVF: A systematic review and meta analysis. *Gynecol Endocrinol.* 2013;29:187–91.
81. Xiao JS, Su CM, Zeng XT. Comparisons of GnRH antagonist versus GnRH agonist protocol in supposed normal ovarian responders undergoing IVF: A systematic review and meta-analysis. *PLoS ONE.* 2014;9:e106854.
82. Al-Inany HG, Youssef MA, Aboulghar M, Broekmans FJ, Sterrenburg MD, Smit JG, et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev.* 2011;(5):CD001750.
83. Weigert M, Krischker U, Pöhl M, Poschalko G, Kindermann C, Feichtinger W. Comparison of stimulation with clomiphene citrate in combination with recombinant follicle-stimulating hormone and recombinant luteinizing hormone to stimulation with a gonadotropin-releasing hormone agonist protocol: A prospective, randomized study. *Fertil Steril.* 2002;78(1):34–9.
84. Karimzadeh MA, Ahmadi S, Oskouian H, Rahmani E. Comparison of mild stimulation and conventional stimulation in ART outcome. *Arch Gynecol Obstet.* 2010 Apr;281(4):741–6.
85. Figueiredo JBP, Nastri CO, Vieira ADD, Martins WP. Clomiphene combined with gonadotropins and GnRH antagonist versus conventional controlled ovarian hyperstimulation without clomiphene in women undergoing assisted reproductive techniques: Systematic review and meta-analysis. *Arch Gynecol Obstet.* 2013;287:779–90.
86. Gibrel A, Maheshwari A, Bhattacharya S. Clomiphene citrate in combination with gonadotropins for controlled ovarian stimulation in women undergoing in vitro fertilization. *Cochrane Database Syst Rev.* 2012 Nov 14;11:CD008528.
87. Galindo A, Bodri D, Guillén JJ, Colodrán M, Vernaeve V, Coll O. Triggering with HCG or GnRH agonist in GnRH antagonist treated oocyte donation cycles: A randomised clinical trial. *Gynecol Endocrinol.* 2009 Jan;25(1):60–6.
88. Melo M, Busso CE, Bellver J, Alama P, Garrido N, Meseguer M, et al. GnRH agonist versus recombinant HCG in an oocyte donation programme: A randomized, prospective, controlled, assessor-blind study. *Reprod Biomed Online.* 2009;19(4):486–92.
89. Engmann L, DiLuigi A, Schmidt D, Nulsen J, Maier D, Benadiva C. The use of gonadotropin-releasing hormone (GnRH) agonist to induce oocyte maturation after cotreatment with GnRH antagonist in high-risk patients undergoing in vitro fertilization prevents the risk of ovarian hyperstimulation syndrome: A prospective randomized controlled study. *Fertil Steril.* 2008 Jan;89(1):84–91.
90. Sismanoglu A, Tekin HI, Erden HF, Ciray NH, Ulug U, Bahceci M. Ovulation triggering with GnRH agonist vs. hCG in the same egg donor population undergoing donor oocyte cycles with GnRH antagonist: A prospective randomized cross-over trial. *J Assist Reprod Genet.* 2009 May;26(5):251–6.
91. Bodri D, Guillén JJ, Polo A, Trullenque M, Esteve C, Coll O. Complications related to ovarian stimulation and oocyte retrieval in 4052 oocyte donor cycles. *Reprod Biomed Online.* 2008;17(2):237–43.
92. Imbar T, Kol S, Lossos F, Bdolah Y, Hurwitz A, Haimov-Kochman R. Reproductive outcome of fresh or frozen-thawed embryo transfer is similar in high-risk patients for ovarian hyperstimulation syndrome using GnRH agonist for final oocyte maturation and intensive luteal support. *Hum Reprod.* 2012;27(3):753–9.

93. Oktay K, Türkçüoğlu I, Rodriguez-Wallberg KA. GnRH agonist trigger for women with breast cancer undergoing fertility preservation by aromatase inhibitor/FSH stimulation. *Reprod Biomed Online*. 2010;20(6):783–8.
94. Orvieto R, Rabinson J, Meltzer S, Zohav E, Anteby E, Homburg R. Substituting HCG with GnRH agonist to trigger final follicular maturation - A retrospective comparison of three different ovarian stimulation protocols. *Reprod Biomed Online*. 2006;13(2):198–201.
95. Youssef MAFM, Van der Veen F, Al-Inany HG, Mochtar MH, Griesinger G, Nagi Mohesen M, et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database Syst Rev*. 2014;2014:CD008046.
96. Castillo JC, Dolz M, Bienvenido E, Abad L, Casan EM, Bonilla-Musoles F. Cycles triggered with GnRH agonist: Exploring low-dose HCG for luteal support. *Reprod Biomed Online*. 2010;20(2):175–81.
97. Humaidan P, Polyzos NP, Alsberg B, Erb K, Mikkelsen AL, Elbaek HO, et al. GnRH trigger and individualized luteal phase hCG support according to ovarian response to stimulation: Two prospective randomized controlled multi-centre studies in IVF patients. *Hum Reprod*. 2013;28(9):2511–21.
98. Revelli A, Dolfin E, Gennarelli G, Lantieri T, Massobrio M, Holte JG, et al. Low-dose acetylsalicylic acid plus prednisolone as an adjuvant treatment in IVF: A prospective, randomized study. *Fertil Steril*. 2008 Nov;90(5):1685–91.
99. Várnagy Á, Bódis J, Mánfai Z, Wilhelm F, Busznyák C, Koppán M. Low-dose aspirin therapy to prevent ovarian hyperstimulation syndrome. *Fertil Steril*. 2010;93(7):2281–4.
100. Tang T, Glanville J, Orsi N, Barth JH, Balen AH. The use of metformin for women with PCOS undergoing IVF treatment. *Hum Reprod*. 2006;21(6):1416–25.
101. Huang X, Wang P, Tal R, Lv F, Li Y, Zhang X. A systematic review and meta-analysis of metformin among patients with polycystic ovary syndrome undergoing assisted reproductive technology procedures. *Int J Gynecol Obstetr*. 2015;131:111–6.
102. Palomba S, Falbo A, La Sala GB. Effects of metformin in women with polycystic ovary syndrome treated with gonadotrophins for in vitro fertilisation and intracytoplasmic sperm injection cycles: A systematic review and meta-analysis of randomised controlled trials. *BJOG*. 2013;120:267–76.
103. Palomba S, Falbo A, Carrillo L, Villani MT, Orio F, Russo T, et al. Metformin reduces risk of ovarian hyperstimulation syndrome in patients with polycystic ovary syndrome during gonadotropin-stimulated in vitro fertilization cycles: A randomized, controlled trial. *Fertil Steril*. 2011;96(6):1384–90.e4.
104. Qublan HS, Al-Khaderei S, Abu-Salem AN, Al-Zpoon A, Al-Khateeb M, Al-Ibrahim N, et al. Metformin in the treatment of clomiphene citrate-resistant women with polycystic ovary syndrome undergoing in vitro fertilisation treatment: A randomised controlled trial. *J Obstet Gynaecol*. 2009;29(7):651–5.
105. Swanton A, Lighten A, Granne I, McVeigh E, Lavery S, Trew G, et al. Do women with ovaries of polycystic morphology without any other features of PCOS benefit from short-term metformin co-treatment during IVF? A double-blind, placebo-controlled, randomized trial. *Hum Reprod*. 2011;26(8):2178–84.
106. Kumbak B, Kahraman S. Efficacy of metformin supplementation during ovarian stimulation of lean PCOS patients undergoing in vitro fertilization. *Acta Obstet Gynecol Scand*. 2009;88(5):563–8.
107. Cenksoy C, Cenksoy PO, Erdem O, Sancak B, Gursoy R. A potential novel strategy, inhibition of vasopressin-induced VEGF secretion by relcovaptan, for decreasing the incidence of ovarian hyperstimulation syndrome in the hyperstimulated rat model. *Eur J Obstet Gynecol Reprod Biol*. 2014;174(1):86–90.
108. Nardo LG, Cheema P, Gelbaya TA, Horne G, Fitzgerald CT, Pease EHE, et al. The optimal length of "coasting protocol" in women at risk of ovarian hyperstimulation syndrome undergoing in vitro fertilization. *Hum Fertil*. 2006 Sep;9(3):175–80.
109. Dhont M, Van Der Straeten F, De Sutter P. Prevention of severe ovarian hyperstimulation by coasting. *Fertil Steril*. 1998 Nov;70(5):847–50.
110. Gera PS, Tatpati LL, Allemand MC, Wentworth MA, Coddington CC. Ovarian hyperstimulation syndrome: Steps to maximize success and minimize effect for assisted reproductive outcome. *Fertil Steril*. 2010 Jun;94(1):173–8.
111. Chen C, Der, Chao KH, Yang JH, Chen SU, Ho HN, Yang YS. Comparison of coasting and intravenous albumin in the prevention of ovarian hyperstimulation syndrome. *Fertil Steril*. 2003 Jul 1;80(1):86–90.
112. Egbase PE, Sharhan MA, Grudzinskas JG. Early unilateral follicular aspiration compared with coasting for the prevention of severe ovarian hyperstimulation syndrome: A prospective randomized study. *Hum Reprod*. 1999;14(6):1421–5.
113. D'Angelo A, Brown J, Amso NN. Coasting (withholding gonadotrophins) for preventing ovarian hyperstimulation syndrome. *Cochrane Database Syst Rev*. 2011;(6):CD002811.
114. Lee C, Tummon I, Martin J, Nisker J, Power S, Tekpetey F. Does withholding gonadotrophin administration prevent severe ovarian hyperstimulation syndrome? *Hum Reprod*. 1998;13(5):1157–8.
115. Tehraninejad ES, Hafezi M, Arabipoor A, Aziminekoo E, Chehrazi M, Bahmanabadi A. Comparison of cabergoline and intravenous albumin in the prevention of ovarian hyperstimulation syndrome: A randomized clinical trial. *J Assist Reprod Genet*. 2012 Mar;29(3):259–64.
116. Álvarez C, Martí-Bonmatí L, Novella-Maestre E, Sanz R, Gómez R, Fernández-Sánchez M, et al. Dopamine agonist cabergoline reduces hemoconcentration and ascites in hyperstimulated women undergoing assisted reproduction. *J Clin Endocrinol Metab*. 2007;92(8):2931–7.
117. Carizza C, Abdelmassih V, Abdelmassih S, Ravizzini P, Salgueiro L, Salgueiro PT, et al. Cabergoline reduces the early onset of ovarian hyperstimulation syndrome: A prospective randomized study. *Reprod Biomed Online*. 2008;17(6):751–5.
118. Matorras R, Andrés M, Mendoza R, Prieto B, Pijoan JI, Expósito A. Prevention of ovarian hyperstimulation syndrome in GnRH agonist IVF cycles in moderate risk patients: Randomized study comparing hydroxyethyl starch versus cabergoline and hydroxyethyl starch. *Eur J Obstet Gynecol Reprod Biol*. 2013;170(2):439–43.
119. Seow KM, Lin YH, Bai CH, Chen HJ, Hsieh BC, Huang LW, et al. Clinical outcome according to timing of cabergoline initiation for prevention of OHSS: A randomized controlled trial. *Reprod Biomed Online*. 2013;26(6):562–8.
120. S A, S A, Y MA. Can dopamine agonist at a low dose reduce ovarian hyperstimulation syndrome in women at risk undergoing ICSI treatment cycles? A randomized controlled study. *Eur J Obstet Gynecol Reprod Biol*. 2012;165(2):254–8.
121. Tang H, Mourad SM, Wang A, Zhai S, Di, Hart RJ. Dopamine agonists for preventing ovarian hyperstimulation syndrome. *Cochrane Database Syst Rev*. 2021;2021: CD008605.
122. Rubenfeld ES, Dahan MH. Does the timing of cabergoline administration impact rates of ovarian hyperstimulation syndrome? *Obstet Gynecol Sci*. 2021;64(4):345–52.
123. Leitao VMS, Moroni RM, Seko LMD, Nastri CO, Martins WP. Cabergoline for the prevention of ovarian hyperstimulation syndrome: Systematic review and meta-analysis of randomized controlled trials. *Fertil Steril*. 2014;101(3):664–75.
124. Isikoglu M, Berkkanoglu M, Senturk Z, Ozgur K. Human albumin does not prevent ovarian hyperstimulation syndrome in assisted reproductive technology program: A prospective randomized placebo-controlled double blind study. *Fertil Steril*. 2007 Oct;88(4):982–5.
125. Shalev E, Giladi Y, Matilsky M, Ben-ami M. Decreased incidence of severe ovarian hyperstimulation syndrome in high risk in-vitro fertilization patients receiving intravenous albumin: A prospective study. *Hum Reprod*. 1995 Jun;10(6):1373–86.

126. Shoham Z, Weissman A, Barash A, Borenstein R, Schachter M, Insler V. Intravenous albumin for the prevention of severe ovarian hyperstimulation syndrome in an in vitro fertilization program: A prospective, randomized, placebo-controlled study. *Fertil Steril.* 1994;62(1):137–42.
127. Ben-Chetrit A, Eldar-Geva T, Gal M, Huerta M, Mimon T, Algur N, et al. The questionable use of albumin for the prevention of ovarian hyperstimulation syndrome in an IVF programme: A randomized placebo-controlled trial. *Hum Reprod.* 2001;16(9):1880–4.
128. Bellver J, Muñoz EA, Ballesteros A, Soares SR, Bosch E, Simón C, et al. Intravenous albumin does not prevent moderate-severe ovarian hyperstimulation syndrome in high-risk IVF patients: A randomized controlled study. *Hum Reprod.* 2003;18(11):2283–8.
129. Jee BC, Suh CS, Kim YB, Kim SH, Choi YM, Kim JG, et al. Administration of intravenous albumin around the time of oocyte retrieval reduces pregnancy rate without preventing ovarian hyperstimulation syndrome: A systematic review and meta-analysis. *Gynecol Obstet Invest.* 2010;70:47–54.
130. Venetis CA, Kolibianakis EM, Toulis KA, Goulis DG, Papadimas I, Tarlatzis BC. Intravenous albumin administration for the prevention of severe ovarian hyperstimulation syndrome: A systematic review and metaanalysis. *Fertil Steril.* 2011;95(1):188–96, 196.e1–3.
131. Youssef MA, Al-Inany HG, Evers JL, Aboulghar M. Intra-venous fluids for the prevention of severe ovarian hyperstimulation syndrome. *Cochrane Database Syst Rev.* 2011;(2):CD001302.
132. El-Khayat W, Elsadek M. Calcium infusion for the prevention of ovarian hyperstimulation syndrome: A double-blind randomized controlled trial. *Fertil Steril.* 2015;103(1):101–5.
133. Gurgan T, Demirok A, Guven S, Benkhalfia M, Girgin B, Li TC. Intravenous calcium infusion as a novel preventive therapy of ovarian hyperstimulation syndrome for patients with polycystic ovarian syndrome. *Fertil Steril.* 2011 Jul;96(1):53–7.
134. Naredi N, Karunakaran S. Calcium gluconate infusion is as effective as the vascular endothelial growth factor antagonist cabergoline for the prevention of ovarian hyperstimulation syndrome. *J Hum Reprod Sci.* 2013;6(4):248.
135. Shaker AG, Zosmer A, Dean N, Bekir JS, Jacobs HS, Tan SL. Comparison of intravenous albumin and transfer of fresh embryos with cryopreservation of all embryos for subsequent transfer in prevention of ovarian hyperstimulation syndrome. *Fertil Steril.* 1996;65(5):992–6.
136. Ferraretti AP, Gianaroli L, Magli C, Fortini D, Selman HA, Feliciani E. Elective cryopreservation of all pronucleate embryos in women at risk of ovarian hyperstimulation syndrome: Efficiency and safety. *Hum Reprod.* 1999;14(6):1457–60.
137. D'Angelo A, Amso N. Embryo freezing for preventing ovarian hyperstimulation syndrome. *Cochrane Database Syst Rev.* 2007;(3):CD002806.
138. Rotshenker-Olshinka K, Badeghiesh A, Volodarsky-Perel A, Steiner N, Suarthana E, Dahan MH. Trends in ovarian hyperstimulation syndrome hospitalization rates in the USA: An ongoing concern. *Reprod Biomed Online.* 2020;41:357–60.
139. Fauer BCJM, Nargund G, Andersen AN, Norman R, Tarlatzis B, Boivin J, et al. Mild ovarian stimulation for IVF: 10 years later. *Hum Reprod.* 2010;25:2678–84.
140. Lim KS, Chae SJ, Choo CW, Ku YH, Lee HJ, Hur CY, et al. In vitro maturation: Clinical applications. *Clin Exp Reprod Med.* 2013;40:143–7.
141. Son WY, Yoon SH, Lim JH. Effect of gonadotrophin priming on in-vitro maturation of oocytes collected from women at risk of OHSS. *Reprod Biomed Online.* 2006;13(3):340–8.
142. Aboulghar M. Prediction of ovarian hyperstimulation syndrome (OHSS): Estradiol level has an important role in the prediction of OHSS. *Hum Reprod.* 2003;18:1140–1.
143. Garcia-Velasco JA, Moreno L, Pacheco A, Guillén A, Duque L, Requena A, et al. The aromatase inhibitor letrozole increases the concentration of intraovarian androgens and improves in vitro fertilization outcome in low responder patients: A pilot study. *Fertil Steril.* 2005 Jul;84(1):82–7.
144. Garcia-Velasco JA, Quea G, Piró M, Mayoral M, Ruiz M, Toribio M, et al. Letrozole administration during the luteal phase after ovarian stimulation impacts corpus luteum function: A randomized, placebo-controlled trial. *Fertil Steril.* 2009 Jul;92(1):222–5.
145. Fatemi HM, Popovic-Todorovic B, Donoso P, Papanikolaou E, Smitz J, Devroey P. Luteal phase oestradiol suppression by letrozole: A pilot study in oocyte donors. *Reprod Biomed Online.* 2008;17(3):307–11.
146. Mai Q, Hu X, Yang G, Luo Y, Huang K, Yuan Y, et al. Effect of letrozole on moderate and severe early-onset ovarian hyperstimulation syndrome in high-risk women: A prospective randomized trial. *Am J Obstet Gynecol.* 2017 Jan 1;216(1):42.e1–42.e10.
147. He Q, Liang L, Zhang C, Li H, Ge Z, Wang L, et al. Effects of different doses of letrozole on the incidence of early-onset ovarian hyperstimulation syndrome after oocyte retrieval. *Syst Biol Reprod Med.* 2014 Dec 1;60(6):355–60.
148. Wang YQ, Wang J, Wang WM, Xie QZ, Yan WJ, et al. [Luteal letrozole administration decreases serum estrogen level but not the risk of ovarian hyperstimulation syndrome]. *Beijing Da Xue Bao Yi Xue Ban.* 2013 Dec 18;45(6):869–72.
149. Wang YQ, Luo J, Xu WM, Xie QZ, Yan WJ, Wu GX, et al. Can steroid ovarian suppression during the luteal phase after oocyte retrieval reduce the risk of severe OHSS? *J Ovarian Res.* 2015 Sep 23;8(1).
150. Zhao J, Xu B, Huang X, Yan Y, Li Y. Whether Letrozole could reduce the incidence of early ovary hyperstimulation syndrome after assisted reproductive technology? A systematic review and meta-analysis. *Reprod Health.* 2020 Dec 1;17(1):181.
151. Guardo D, Midassi F, Racca H, Tournaye A, Vos D, Blockeel M. Luteal phase support in IVF: Comparison between evidence-based medicine and real-life practices. *Front Endocrinol (Lausanne).* 2020 Aug 18;11:500.
152. Vaisbuch E, De Ziegler D, Leong M, Weissman A, Shoham Z. Luteal-phase support in assisted reproduction treatment: Real-life practices reported worldwide by an updated website-based survey. *Reprod Biomed Online.* 2014;28(3):330–5.
153. van der Linden M, Buckingham K, Farquhar C, Kremer JAM, Metwally M. Luteal phase support for assisted reproduction cycles. *Cochrane Database Syst Rev.* 2015;2015(7):CD009154.
154. Ludwig M, Diedrich K. Evaluation of an optimal luteal phase support protocol in IVF. *Acta Obstet Gynecol Scand.* 2001;80(5):452–66.
155. Daya S, Gunby J. Luteal phase support in assisted reproduction cycles. *Cochrane Database Syst Rev.* 2004;(3):CD004830.
156. Bar-Hava I, Mizrachi Y, Karfunkel-Doron D, Omer Y, Sheena L, Carmon N, et al. Intranasal gonadotropin-releasing hormone agonist (GnRHa) for luteal-phase support following GnRHa triggering, a novel approach to avoid ovarian hyperstimulation syndrome in high responders. *Fertil Steril.* 2016 Aug 1;106(2):330–3.
157. Friedler S, Grin L. Luteal phase support with GnRH agonist does not eliminate the risk for ovarian hyperstimulation syndrome. *Gynecol Endocrinol.* 2019 May 4;35(5):368–9.
158. Tsafrir A, Weissman A. In reply to, Christ J, Herndon CN, Yu B. Severe ovarian hyperstimulation syndrome associated with long-acting GnRH agonist in oncofertility patients. *J Assist Reprod Genet.* 2021;38:751–6. doi:[10.1007/s10815-020-02051](https://doi.org/10.1007/s10815-020-02051). *J Assist Reprod Genet.* 2021;38(10):2801–2.

BLEEDING, SEVERE PELVIC INFECTION, AND ECTOPIC PREGNANCY

Raoul Orvieto

Transvaginal ultrasound-guided aspiration of oocytes is a well-accepted and universally used method in assisted reproduction [1, 2]. Its major advantages include easy access to ovarian follicles with excellent oocyte yield and good visualization of the major pelvic vessels. It is done as a day care procedure, either under intravenous analgesia and sedation or under general anaesthesia, and is usually atraumatic. Nevertheless, there are some inherent risks, namely puncture of blood vessels and hemoperitoneum, bleeding from the vaginal vault puncture site, rupture of adnexal cystic masses, bladder injury, bowel perforation, trauma to pelvic organs, and pelvic infection. In addition, embryo transfer (ET) itself may be associated with complications such as pelvic infection, multiple pregnancy (which is directly related to the number of transferred embryos), spontaneous abortion, and extrauterine pregnancy (EUP). Maxwell et al. [3] have reported on the incidence of both serious and minor complications in young women undergoing 886 oocyte retrievals for oocyte donation. While the rate of serious complications, which included ovarian hyperstimulation syndrome, ovarian torsion, infection, and ruptured ovarian cyst, was 0.7%, the rate of minor complications severe enough to prompt the donor to seek medical attention after retrieval was 8.5%. A study by Levi-Setti et al. [4] has assessed the incidence of complications necessitating hospitalization or outpatient management following transvaginal oocyte retrieval in a large assisted reproductive technology (ART) programme. The most important, identifiable, risk factors for the occurrence of complications were high number of oocytes retrieved, a long duration of the procedure and mean time per oocyte retrieved, inexperience of the surgeon, younger patients with a lesser BMI, and history of prior abdominal or pelvic surgery or pelvic inflammatory disease (PID).

The aim of the present review is to discuss comprehensively three of these complications: bleeding, PID, and EUP.

Bleeding

Vaginal bleeding

During ultrasound-guided transvaginal oocyte aspiration, multiple punctures of the vaginal vault or inappropriate handling and rotation of the ultrasound vaginal probe while inserting an aspiration needle through the vaginal vault can injure or tear the vaginal mucosa, ovaries, intra-abdominal organs, or blood vessels [1, 5–9]. Bleeding from the vaginal vault is a common consequence of oocyte pickup (OPU), with a reported incidence of 1.4%–18.4% [6]. In most cases, vaginal bleeding as a result of OPU stops spontaneously at the end of the procedure [7]. In cases in which it does not, the bleeding site needs to be identified by vaginal exploration with a large speculum, followed by application of pressure with sponge forceps or vaginal packing with a large gauze roll. If this is unsuccessful or the tear is wide and deep, suturing is necessary. The use of a thinner-tipped needle (0.9 mm in diameter) during OPU resulted in significantly less vaginal bleeding when compared to a standard needle (1.4 mm in diameter) [8].

Intraperitoneal or retroperitoneal bleeding

Transvaginal oocyte aspiration can also cause bleeding if intraperitoneal or retroperitoneal pelvic blood vessels are injured or if there is damage to the fine vascular network surrounding the punctured ovarian follicle. The reported incidence of severe intra- or retro-peritoneal bleeding varies from 0% to 1.3% [1, 7, 9–11]; a recent report described one case of intra-abdominal bleeding complicating the aspiration of 1000 oocyte donors [12]. Young, lean patients and those with polycystic ovary syndrome or a history of previous surgery were specifically demonstrated to be at much higher risk of this complication [13, 14]. Intraperitoneal bleeding tends to be severe with acute hemodynamic deterioration, whereas retroperitoneal bleeding usually has a later and more indolent presentation. Yih et al. [15] studied serial complete blood counts before and after OPU in 93 *in vitro* fertilization (IVF) cycles and demonstrated a non-significant change in haematocrit levels, indicating that clinically significant blood loss after OPU is actually uncommon.

Azem et al. [16] described a patient who presented to the emergency room 10 hours after OPU with severe lower abdominal pain, vomiting, and tenesmus. Examination revealed a distended abdomen with severe tenderness in the pouch of Douglas; on transvaginal sonography, a minimal, 3- to 4-cm collection of fluid was noted. Laparoscopy followed by laparotomy, which was performed on the basis of the clinical profile, revealed a retroperitoneal hematoma 7 cm in diameter. After evacuation and haemostasis, active bleeding from the mid-sacral vein occurred and was controlled by a metal clip. This case demonstrates the indolent course of retroperitoneal bleeding and physicians should be alerted to the possibility of retroperitoneal hematoma despite an absence of free fluid in the pouch of Douglas. Notably, a similar case with no significant intraperitoneal fluid collection was also described as a result of ureteral injury with the consequent uroretroperitoneum [17].

Intra-abdominal bleeding should be suspected immediately after OPU upon the development of signs and symptoms of anaemia—specifically weakness, dizziness, dyspnoea, or persistent tachycardia. Early management consists of intense hemodynamic monitoring, together with serial measurement of blood haemoglobin concentrations and ultrasonographic evaluation for the presence of intra-abdominal fluid. It should be emphasized, however, that intra-abdominal blood clots or retroperitoneal bleeding might be invisible even to an experienced ultrasound operator. A drop in haemoglobin concentration is an indication for prompt blood transfusion. If hemodynamic deterioration continues or acute abdominal pain develops, diagnostic laparoscopy or exploratory laparotomy with subsequent haemostasis of the bleeding site(s) is required. The clinician must make sure to handle the fragile hyperstimulated ovaries very cautiously. Notably, a longer time interval between OPU and surgical intervention was noted to put the patient at risk of ovariectomy [18].

Dicker et al. described three cases of severe intraabdominal bleeding from ovarian puncture sites during OPU, leading to acute abdominal complications [1]. In two of the patients, symptoms developed three hours after OPU (haemoglobin 9.0 g/100 mL and 8.1 g/100 mL, respectively), and laparoscopic drainage and haemostasis were sufficient. The third patient became symptomatic after four hours (haemoglobin 7.3 g/100 mL) and required exploratory laparotomy and haemostasis in addition to the transfusion of four units of blood as a life-saving procedure. Later, Battaglia et al. [19] reported severe intraabdominal bleeding from the surfaces of both ovaries in a patient with coagulation factor XI deficiency. As expected, the patient became symptomatic three hours after OPU and required laparotomy, partial resection of stuffed ovaries, and haemostasis. Physicians should be aware of the presence of concomitant coagulopathy and might therefore consider intense coagulation factor replacement before or during abdominal exploration.

Massive delayed intra-abdominal haemorrhage was also reported following OPU (two and four days later) in patients at risk of thromboembolic events who concomitantly used a therapeutic dose of low-molecular-weight heparin [20]. These cases should direct physicians' attention and keep them alert while conducting an IVF treatment in this subgroup of patients. Moreover, the authors recommended that the patient be kept in the ward for observation for at least two to four days following OPU.

Can we prevent severe intra-abdominal bleeding from ovarian puncture sites during OPU? In a cross-sectional retrospective study, Revel et al. [21] questioned the utility of coagulation screening before OPU. Among the 1032 patients evaluated, they found that 534 coagulation tests were needed to prevent one case of bleeding associated with an abnormal coagulation test result. Moreover, while the use of colour Doppler sonography during OPU was suggested to reduce the risk of blood vessel injury by the guiding needle [22], its routine use could not predict all cases with moderate peritoneal bleeding [23].

Patients with bleeding disorders or those at risk to develop intra-abdominal bleeding might benefit from prophylactic intravenously administration of 1 gr tranexamic acid [24]. Description of the intraoperative measures needed to control intra-abdominal haemorrhage is beyond the scope of this text, and the reader is referred elsewhere for a detailed review [25].

Pelvic inflammatory disease

PID is an infrequent complication of ultrasound-guided transvaginal aspiration of oocytes or ET, with a reported incidence of 0.2%–0.5% per cycle [10, 26–28]. Signs or symptoms of pelvic infection, such as pyrexia, continuous low abdominal pain, dysuria, or offensive vaginal discharge, are infrequent [26]. However, this does not exclude occult, subclinical bacterial colonization, which may influence the success of the IVF–ET treatment, or slowly progress throughout pregnancy [29]. Ashkenazi et al. evaluated the outcomes of all IVF–ET procedures performed in their unit between 1986 and 1992 [28]. Of the 4771 patients who underwent transvaginal OPU, 28 (0.58%) had symptoms of PID within one to seven days. The diagnosis was established by a rise in body temperature to 38°C for more than 48 hours, signs of pelvic peritonitis on physical examination, leukocyte counts of >12,000 cells/m³, and elevated erythrocyte sedimentation rates. All patients were admitted to hospital for treatment with intravenous antibiotics. Notably, ovarian abscess following oocyte retrieval may manifest late

during pregnancy with low-grade fever or vague abdominal pain [30].

OPU can also lead to severe abdominal complications. Our group reported on nine patients (0.24%) with tubo-ovarian or pelvic abscess after transvaginal-guided OPU [28]. Three patients required laparotomy and adnexitomy, whereas in six patients, culdocentesis was performed for adequate pelvic abscess drainage. Kelada and Ghani [31] have described a case of bilateral ovarian abscesses following transvaginal oocyte retrieval, complicated by early signs of consumption coagulopathy. The latter is a serious and life-threatening complication of pelvic infection and sepsis, which should be diagnosed and corrected immediately.

Mechanisms underlying pelvic infection

During transvaginal aspiration, accidental needle transport of cervicovaginal flora into ovarian tissue can cause unilateral or bilateral oophoritis, and accidental puncture of a contaminated or sterile hydrosalpinx can cause salpingitis. Some authors have attributed pelvic infection to infected endometriotic cysts or tubo-ovarian abscesses after aspiration of endometriomas [32, 33] or, rarely, to inadvertent puncture of the bowel. Pelvic infection in women with endometriosis was shown to be more serious and resistant to antibiotic treatment, and frequently required surgical intervention [34]. Pelvic infection can occur as a direct consequence of transcervical ET. This is evidenced by reported cases of PID following ET in an agonadal donor egg recipient [35], or during cryopreserved ET [36]; it may also occur as a result of the reaction of a silent or persistent subclinical infection, as seen occasionally after hysterosalpingography. Another possible cause during ET is catheterization of the uterus, which may force bacteria-laden air or fluid into one or both tubes by a piston-like effect.

Effect of acute pelvic infection on IVF–ET outcome

The first study of the impact of pelvic infection on IVF–ET outcome was reported by Ashkenazi et al. in 1994 [28]. We found that the number of oocytes recovered, fertilized, and cleaved in 28 patients undergoing IVF in whom PID developed was similar to that of a comparison group with mechanical infertility. However, there were no pregnancies in the PID group, as compared with the 23%–31% pregnancy rate per transfer in the whole group of patients treated by IVF, indicating that the appearance of PID at the critical time of implantation may cause a failure to conceive. This finding has several possible explanations, as outlined in detail later in the chapter.

Endotoxemia

Endotoxin-releasing bacteria can be introduced into the peritoneal cavity during transvaginal oocyte recovery and into the uterine cavity or tubes during ET. Ng et al. [37] described a case in which human oocytes were degenerated and fragmented, with no evidence of fertilization, in the presence of *Klebsiella*-derived endotoxin. In a study of the effects of endotoxin infusion on the circulating levels of eicosanoids, progesterone, and cortisol and on abortions, Giri et al. [38] found that first-trimester cows were more sensitive to the abortifacient effect of endotoxin than second- and third-trimester cows. The mechanism of the endotoxin-induced abortion apparently involved the prolonged release of prostaglandin F2α, which has a stimulant effect on uterine smooth muscle contractions and a luteolytic effect resulting in a gradual decline in the plasma level of progesterone [38]. In addition, high endotoxin doses can induce the release of various autacoids, catecholamines, and cortisol, which directly or indirectly

lead to metabolic and circulatory failures and, thereby, termination of pregnancy.

Local inflammatory reaction

Bacteria trigger a chain of events that lead to the activation, proliferation, and differentiation of lymphocytes, and the production of specific antibodies and various cytokines. This excessive production of cytokines may disrupt the delicate balance between the immune and reproductive systems and result in reproductive failure [39–41].

Temperature elevation

Apart from their direct role on implantation and early embryonic development, cytokines may mediate temperature elevation and indirectly affect the outcome of IVF–ET. The febrile reaction is an integrated endocrine, autonomic, and behavioural response coordinated by the hypothalamus. The actions of circulating cytokines, such as interleukin-1 and tumour necrosis factor, on the central nervous system result in the secretion of prostaglandin E2, which initiates an elevation in body temperature together with corticosteroid secretion [42], which is also a component of the stress response. Some authors have suggested that fever is essential for amplifying the emergence of T-cell immunity in peripheral tissues [43]. *In vitro* experiments have shown that temperature elevation leads to disintegration of the cytoskeleton [44] and may affect the transport of organelles. In pregnancy, maternal heat exposure can cause intracellular embryonic damage [45] and inhibit cell mitosis, proliferation, and migration, resulting in cell death. In a study of guinea pig embryos, Edwards et al. [46] reported cell damage within minutes and cell death within hours after heating. Other mechanisms of heat-induced cell injury are microvascular lesions, placental necrosis, and placental infarction [47].

Treatment

The role of prophylactic antibiotics in IVF–ET

The potential for intraperitoneal bacterial contamination during transvaginal oocyte recovery is well known and has led to the routine use of prophylactic antibiotics and vaginal disinfection [48]. Meldrum [49] found no cases of pelvic infection among 88 transvaginal retrievals with the use of intravenous cefazolin and vaginal preparation with povidone–iodine and saline irrigation; nor did Tsai et al. [50] in patients with ovarian endometrioma using only vaginal douching with aqueous povidone–iodine followed by normal saline irrigation. Borlum and Maiggard [51] reported two cases of serious pelvic infection in almost 400 transvaginal aspirations. They used only two vaginal douchings with sterile saline and noted that minimizing the number of repeated vaginal penetrations may have helped with lowering the risk of infection. However, the appropriate type of antibiotic administration, the timing or duration of therapy, and the efficacy of therapy have not yet been established [49, 52]. Indeed, some authors claim that these measures may not only further reduce the incidence of PID after oocyte retrieval, but can even increase the risks of both an adverse reaction and of colonization with resistant organisms. Our experience with vaginal douchings with sterile saline in approximately 1100–1200 OPUs per year revealed a very low rate of PID after OPU. Peters et al. [53] suggested that only women with a tubal abnormality and a history of pelvic infection should receive prophylactic antibiotics before oocyte aspiration, and also possibly after ET. Others have suggested that such patients may benefit from transabdominal or transvesical rather than transvaginal procedures [54, 55].

It is also noteworthy that Egbase et al. [56], in a study of the effects of prophylactic antibiotics in OPU on the endocervical microbial inoculation of the endometrium during ET, found that prophylactic antibiotics not only reduced the number of positive microbiology cultures of embryo catheter tips, but also significantly increased implantation and clinical pregnancy rates. On the other hand, in their prospective randomized study, Peikrishvili et al. [57] could not demonstrate any beneficial effects of antibiotic prescription (amoxicillin + clavulanic acid 1 g/125 mg) for six days following oocyte retrieval on implantation, pregnancy, or miscarriage rates.

Curative

PID or tubo-ovarian abscesses after OPU require accurate diagnosis and prompt treatment with broad-spectrum antibiotics. In the presence of a pelvic abscess that is larger than 8 cm or unresponsive to medication, transvaginal or percutaneous drainage is the treatment of choice [48], with or without ultrasound-guided intracavitary instillation of a combination of antibiotics [58]. Patients who received antibiotics alone are more likely to require further surgical intervention when compared with patients who additionally received image-guided drainage [59]. Sometimes, surgical laparoscopy or laparotomy is needed to evacuate the abscess or remove the infected tubes or adnexa.

Summary

The appearance of PID at the critical time of implantation results in failure to conceive. This effect may be mediated by bacterial endotoxins, a local inflammatory reaction against bacteria with the involvement of cytokines that affects implantation and early embryonic development, or temperature elevation that directly affects the conceptus. Although the role of prophylactic antibiotics is still controversial, they can be considered in the presence of risk factors for PID; aspiration of hydrosalpinx or endometriomas during OPU might be a risk factor for infection and should be avoided. Furthermore, to prevent total failure, if PID develops before ET, cryopreservation and ET in subsequent cycles should be considered. However, if PID develops after ET, the bacterial infection and fever should be treated rigorously to prevent reproductive failure.

Extrauterine pregnancy

EUP is the implantation of a blastocyst anywhere except in the endometrial lining of the uterine cavity. In recent years, EUPs have shown a marked increase in both absolute number and rate of occurrence [60]. By 1992, almost 2% of all pregnancies in the United States were EUPs, and ectopic pregnancies accounted for 10% of all pregnancy-related deaths [60, 61]. The rates of abortions, multiple pregnancies, and EUPs are higher in pregnancies resulting from ART than in spontaneous pregnancies.

Other factors associated with the development of EUP include previous EUP, salpingitis, previous surgery to the fallopian tube, peritubal adhesions, pelvic lesions that distort the tube, developmental abnormalities of the tube, and altered tubal motility.

EUP after ART

The first IVF–ET pregnancy reported was an ectopic pregnancy [62]. Today, the incidence of EUPs after IVF ranges from 2.1% to 9.4% of all clinical pregnancies [63, 64]. In 2007, the Society for Assisted Reproductive Technology (SART) [65] reported an incidence of EUPs of 1.8% of all pregnancies, compared with 1.6% in

1996. This finding was attributable to the decrease in the proportion of couples with tubal factor infertility undergoing IVF treatment and a concomitant increase in couples with male factor infertility. Later, the SART reported the outcomes of ART initiated in the United States in 2001 [65]. The incidence of EUP for all ART procedures was 0.8% per transfer and 1.6% per clinical pregnancy, which compares favourably with the estimated overall incidence of EUP in the United States of 2% per reported pregnancy [60]. Perkins et al. assessed the risk of EUP associated with ART in the United States between 2001 and 2011. While a decline in the incidence of EUP was observed over the study period, with the most pronounced decline seen with frozen ETs, multiple ETs increase the risk of ectopic pregnancy [66].

Risk factors

Data on risk factors for EUP after IVF are still unclear. Martinez and Trounson [67] failed to identify any risk factors, whereas Karande et al. [68] pointed to a prior ectopic pregnancy. Verhulst et al. [69] found a significantly higher rate of EUP after IVF in patients with tubal disease (3.6%) compared with those with normal tubes (1.2%); this finding was confirmed by several other studies [64, 70–72]. Cohen et al. [73] showed that the number of patent tubes at the time of transfer was a risk factor, with a higher EUP rate in patients with zero or two patent tubes than in patients with one. In an analysis of the Bourn Hall Clinic data, Marcus and Brinsden [74] noted that the main risk factor was a history of PID. Though they found EUP to be more prevalent in patients with tubal factor infertility, those who received a higher culture medium volume and those with a higher progesterone/oestradiol ratio on the day of ET had no associated history of EUP. Acharya et al. found that an increased oocyte yield correlated with a significantly increased EUP rate [75]. Since this association was not found in oocyte recipients, they suggested that this increased EUP rate may be related to the supraphysiologic hormone levels achieved during ovarian stimulation [75]. Finally, in a meta-analysis of risk factors for EUP, Ankum et al. [76] concluded that the four most significant were previous EUP, documented tubal pathology, previous tubal surgery, and in utero exposure to diethylstilboestrol. These results were confirmed by Lesny et al., who also added one more: a difficult ET on day 2 rather than day 3 [77]. Clayton et al. [78] have analysed the EUP risk among 94,118 patients who conceived with ART procedures. A total of 2009 (2.1%) were ectopic. In comparison with the ectopic rate (2.2%) among pregnancies conceived with IVF (fresh, non-donor cycles), the ectopic rate was significantly increased when zygote intrafallopian transfer was used (3.6%) and significantly decreased when donor oocytes were used (1.4%) or when a gestational surrogate carried the pregnancy (0.9%). Among fresh nondonor IVF-ET procedures, the risk of ectopic pregnancy was significantly increased among women with tubal factor infertility, endometriosis, and other non-tubal female factors of infertility, and significantly decreased among women with a previous live birth. Moreover, transfer of high-quality embryos was associated with a decreased ectopic risk when two or fewer embryos were transferred, but not when three or more embryos were transferred. By analysing the SART database from 2008 to 2011, Londra et al. [79] found that the odds of EUP were 65% lower in women who had a frozen compared with a fresh transfer in autologous cycles. Moreover, frozen-thawed day-5 blastocyst transfer was associated with a lower EUP rate than frozen-thawed day-3 transfer and fresh transfer [80, 81]. Liu et al. [82] investigated the influence of endometrial thickness on the incidence of EUP in FET cycles.

After adjusting for confounders, endometrial thickness remained statistically significant as an independent risk factor for EUP. Compared with women with an endometrial thickness of ≥ 14 mm, the adjusted odds ratio (aORs) for women with endometrial thickness in the ranges 7–7.9, 8–9.9, and 10–11.9 mm were 2.70, 2.06, and 1.66, respectively. Hormone replacement treatment for endometrial preparation during FET increased the risk of EUP after adjustment for confounding variables. There are many theories on the manner by which embryos implant in the fallopian tube following ET: by the hydrostatic force of the transfer medium containing the embryos in the fallopian tube ostia; by the gravitational pull of the embryos to the hanging tubes, which are located lower than the uterine fundus; or by reflux expulsion of the embryo due to embryonic migration to the fallopian tubes, either spontaneously or secondary to uterine contractions [83]. The technique of ET itself may also be a culprit in EUP, although this is controversial [84]. For example, while Yovich et al. [85] noted a significantly higher rate of EUP when the embryos were placed high near the uterine fundus or into the tube itself, rather than in the lower uterus, Friedman et al. [86] have demonstrated that blastocyst transfer closer to the fundus (<10 mm) is associated with a higher pregnancy rate. However, although in the latter study no EUP occurred in the <10 -mm group, this outcome should be monitored closely in larger studies.

The transfer volume of culture media containing embryos may play a role in embryonic migration into the fallopian tubes. While most clinicians contend that more than 80 μ L of media is needed for the embryo to reach the fallopian tube [64], Knutzen et al. [87], using a mock intrauterine ET with 50 μ L of radiopaque dye, demonstrated easy passage of all or part of the material in 44% of patients. Lesny et al. [88] explained these findings as due to the propulsion of the embryo from the uterine fundus into the tubes by the junctional zone contractions. Therefore, as the likelihood of tubal placement is very high, the development of tubal pregnancy is not due solely to embryos reaching the tubes, but rather to an additional pathological process that prevents their movement back into the uterine cavity. Potential mechanisms may involve tubal disease affecting the luminal surface and thereby delaying or blocking embryonic passage into the uterine cavity, external factors that interfere with tubal motility, and abnormal embryos [69], such as those derived from chromosomally abnormal gametes [89]. Refaat et al. [90] reviewed the scientific literature regarding EUP during IVF-ET. A history of tubal infertility, PID and specific aspects of ET technique were the most significant risk factors for later EUP.

To ameliorate the role of abnormal fallopian tubes in the pathogenesis of EUP after IVF, several authors have recommended that the tubes be occluded at the level of the uterotubal junction [91, 92]. However, this measure does not prevent the development of an interstitial pregnancy [73], although it certainly prevents the well-known phenomenon of spontaneous pregnancies after IVF treatment, which occur in 30% of patients with patent tubes [93].

Another potential interfering factor in tubal function and ET is the different hormonal milieus resulting from ovulation induction protocols, particularly those including clomiphene citrate [69, 94]. This may result from the effect of the high oestradiol levels on tubal peristalsis through the control of tubal smooth muscle contractility and ciliary activity [85, 94]. A Japanese study [95] has retrospectively evaluated the risk of EUPs among 68,851 clinical pregnancies, according to different ovarian stimulation protocols. Compared with natural cycles, all ovarian stimulation protocols were associated with a significantly increased risk of

EUP. Ovarian stimulation with clomiphene demonstrated the highest odds ratios for EUPs. Significant associations between ovarian stimulation protocols and EP compared with natural cycles. Pygriots et al. [72], however, did not demonstrate a difference in oestradiol levels on the day of human chorionic gonadotropin (hCG) administration between IVF patients with and without EUP. Furthermore, they found an increased proportion of EUPs in frozen ETs following natural cycles in which the oestradiol levels were comparatively low. Of interest, is the study by Fang et al. [96], evaluating the predictive value of endometrial thickness EUP. A decreased risk of EUP was found among patients with an endometrial thickness >10 mm prior to ET. Moreover, a cut-off value of endometrial thickness for EUP prediction was 10.65 mm, with a sensitivity of 59% and a specificity of 63%.

In summary, the reproductive health characteristics of infertile women, the different hormonal milieus, the technical issues of IVF procedures, and the estimated embryo implantation potential were all suggested as possible risk factors [97]; however, the mechanisms are still uncertain and need further investigation.

Heterotopic pregnancy following ART

The general incidence of combined intrauterine and extrauterine (heterotopic) pregnancy is 1:15,000–30,000, and it increases dramatically to 1:100 in pregnancies following ART or ovulation induction [98–100]. Although a distorted pelvic anatomy is responsible for the predisposition to both extrauterine and heterotopic pregnancy [101–103], heterotopic pregnancies are associated with a greater number of embryos transferred, whereas EUP is not. Tummon et al. [104] reported that when four or more embryos were transferred, the odds ratio for the development of a heterotopic pregnancy versus EUP was 10. The difficult diagnosis of this potentially life-threatening complication is often made during emergency surgery following tubal rupture and hemoperitoneum. In about 70% of cases, the outcome of the intrauterine pregnancy is favourable (live birth) once the EUP is terminated [105, 106]. Since the diagnosis is challenging due to the falsely reassuring presence of an intrauterine fetus, a high index of suspicion and early intervention are mandatory to salvage the viable intrauterine pregnancy and prevent maternal mortality [107].

Diagnosis and treatment

Non-invasive diagnostic measures using transvaginal ultrasonography combined with serum hCG monitoring have proved to be a reliable tool in the diagnosis of EUP. Since most pregnancies following ART are monitored at an early stage before the onset of symptoms, early diagnosis of the condition and improved management and care have resulted in a decline in the morbidity and mortality of EUP. Of note is the fact that treating EUP with methotrexate has no influence on patients' serum anti-Mullerian hormone levels [108], nor patients' performance in the following IVF cycle [109, 110]. The diagnosis and treatment of EUP are beyond the scope of this chapter, and readers are referred elsewhere for detailed reviews [111, 112].

Brief summary

A recent web-based questionnaire, with distinct questions related to the practice of transvaginal OPU, revealed a wide variation in the practices of minimizing infection and bleeding complications [113]. Transvaginal ultrasound-guided aspiration of oocytes is a well-accepted and universally used method in

assisted reproduction. Its major advantages include easy access to ovarian follicles with excellent oocyte yield and good visualization of the major pelvic vessels, and it is usually atraumatic. Nevertheless, there are some inherent risks, namely puncture of blood vessels and intra-abdominal or retroperitoneal bleeding, bleeding from the vaginal vault puncture site, rupture or perforation of pelvic organs, and pelvic infection. In addition, ET itself may be associated with complications such as pelvic infection, multiple pregnancy, or EUP. This chapter has comprehensively presented and discussed three of these complications: bleeding, PID, and EUP.

References

- Dicker D, Ashkenazi J, Feldberg D, et al. Severe abdominal complications after transvaginal ultrasonographically-guided retrieval of oocytes for *in vitro* fertilization and embryo transfer. Fertil Steril. 1993;59:1313–5.
- ESHRE Working Group on Ultrasound in ART, D'Angelo A, Panayotidis C, Amso N, Marci R, Matorras R, Onofrescu M, Turp AB, Vandekerckhove F, Veleva Z, Vermeulen N, Vlaisavljevic V. Recommendations for good practice in ultrasound: Oocyte pick up. Hum Reprod Open. 2019;2019(4):hoz025.
- Maxwell KN, Cholst IN, Rosenwaks Z. The incidence of both serious and minor complications in young women undergoing oocyte donation. Fertil Steril. 2008;90:2165–71.
- Levi-Setti PE, Cirillo F, Sciarro V, Morenghi E, Heilbron F, Girardello D, Zannoni E, Patrizio P. Appraisal of clinical complications after 23,827 oocyte retrievals in a large assisted reproductive technology program. Fertil Steril. 2018;109(6):1038–43.
- Modder J, Kettel LM, Sakamoto K. Hematuria and clot retention after transvaginal oocyte aspiration: A case report. Fertil Steril. 2006;86:e1–2.
- Tureck RW, Garcia C, Blasco L, Mastrianni L. Perioperative complications arising after transvaginal oocyte retrieval. Obstet Gynecol. 1993;81:590–3.
- Lenz S, Leeton J, Renou P. Transvaginal recovery of oocytes for *in vitro* fertilization using vaginal ultrasound. J In Vitro Fert Embryo Transf. 1987;4:51–5.
- Wikland M, Blad S, Bungum L, Hillensjo T, Karlstrom PO, Nilsson S. A randomized controlled study comparing pain experience between a newly designed needle with a thin tip and a standard needle for oocyte aspiration. Hum Reprod. 2011;26:1377–83.
- Serour GI, Aboulghar M, Mansour R, et al. Complications of medically assisted conception in 3500 cycles. Fertil Steril. 1998;70:638–42.
- Govaert I, Devreker F, Delbaere A, et al. Short-term medical complications of 1500 oocyte retrievals for *in vitro* fertilization and embryo transfer. Eur J Obstet Gynecol Reprod Biol. 1998;77:239–43.
- Aragona C, Mohamed MA, Espinola MS, et al. Clinical complications after transvaginal oocyte retrieval in 7,098 IVF cycles. Fertil Steril. 2011;95:293–4.
- Sauer MV. Defining the incidence of serious complications experienced by oocyte donors: A review of 1000 cases. Am J Obstet Gynecol. 2001;184:277–8.
- Liberty G, Hyman JH, Eldar-Geva T, et al. Ovarian hemorrhage after transvaginal ultrasonographically guided oocyte aspiration: A potentially catastrophic and not so rare complication among lean patients with polycystic ovary syndrome. Fertil Steril. 2010;93:874–9.
- Zhen X, Qiao J, Ma C, Fan Y, Liu P. Intraperitoneal bleeding following transvaginal oocyte retrieval. Int J Gynaecol Obstet. 2010;108:31–4.
- Yih MC, Goldschlag D, Davis OK, et al. Complete blood counts (CBC) after oocyte retrieval: What is normal? Fertil Steril. 2001;76:S115–6.

16. Azem F, Wolf Y, Botchan A, et al. Massive retroperitoneal bleeding: A complication of transvaginal ultrasonography-guided oocyte retrieval for *in vitro* fertilization-embryo transfer. *Fertil Steril.* 2000;74:405–6.
17. Fiori O, Cornet D, Darai E, Antoine JM, Bazot M. Uro-retroperitoneum after ultrasound-guided transvaginal follicle puncture in an oocyte donor: A case report. *Hum Reprod.* 2006;21:2969–71.
18. Nouri K, Walch K, Promberger R, Kurz C, Tempfer CB, Ott J. Severe haematoperitoneum caused by ovarian bleeding after transvaginal oocyte retrieval: A retrospective analysis and systematic literature review. *Reprod Biomed Online.* 2014;29:699–707.
19. Battaglia C, Regnani G, Giulini S, et al. Severe intraabdominal bleeding after transvaginal oocyte retrieval for IVF-ET and coagulation factor XI deficiency: A case report. *J Assist Reprod Genet.* 2001;3:178–81.
20. Mashiach R, Stockheim D, Zolti M, Orvieto R. Delayed intra-abdominal bleeding following transvaginal ultrasonography guided oocyte retrieval for *in vitro* fertilization in patients at risk for thrombo-embolic events under anticoagulant therapy. *F1000Res.* 2013;2:189.
21. Revel A, Schejter-Dinur Y, Yahalom SZ, et al. Is routine screening needed for coagulation abnormalities before oocyte retrieval? *Fertil Steril.* 2011;95:1182–4.
22. Shalev J, Orvieto R, Meizner I. Use of color Doppler sonography during follicular aspiration in patients undergoing *in vitro* fertilization may reduce the risk of blood vessel injury. *Fertil Steril.* 2004;81:1408–10.
23. Risquez F, Confino E. Can doppler ultrasoundguided oocyte retrieval improve IVF safety? *Reprod Biomed Online.* 2010;21: 444–5.
24. Sentilhes L, Sénat MV, Le Lous M. Tranexamic acid for the prevention of blood loss after cesarean delivery. *N Engl J Med.* 2021;384: 1623–34.
25. Mutch DG, Kuroki LM. Surgical control of pelvic hemorrhage. In: *Te Linde's Operative Gynecology.* 12th edn. Handa VL, Linda Van Le L (eds). Philadelphia, PA: Walters Kluwer, 2019, pp. 156–72.
26. Howe RS, Wheeler C, Mastroianni L Jr., et al. Pelvic infection after transvaginal ultrasound-guided ovum retrieval. *Fertil Steril.* 1988;49:726–8.
27. Curtis P, Amso N, Keith E, et al. Evaluation of the risk of pelvic infection following transvaginal oocyte recovery. *Hum Reprod.* 1992;7:625–6.
28. Ashkenazi J, Farhi J, Dicker D, et al. Acute pelvic inflammatory disease after oocyte retrieval: Adverse effects on the results of implantation. *Fertil Steril.* 1994;61:526–8.
29. Al-Kuran O, Beitawi S, Al-Mehaisen L. Pelvic abscess complicating an *in vitro* fertilization pregnancy and review of the literature. *J Assist Reprod Genet.* 2008;25:341–3.
30. Sharpe K, Karovitch AJ, Claman P, Suh KN. Transvaginal oocyte retrieval for *in vitro* fertilization complicated by ovarian abscess during pregnancy. *Fertil Steril.* 2006;86:219.e11–3.
31. Kelada E, Ghani R. Bilateral ovarian abscesses following transvaginal oocyte retrieval for IVF: A case report and review of literature. *J Assist Reprod Genet.* 2007;24:143–5.
32. Yaron Y, Peyser MR, Samuel D, et al. Infected endometriotic cysts secondary to oocyte aspiration for *in vitro* fertilization. *Hum Reprod.* 1994;9:1759–60.
33. Nargund G, Parsons J. Infected endometriotic cysts secondary to oocyte aspiration for *in vitro* fertilization. *Hum Reprod.* 1995;10:1555.
34. Elizur SE, Lebovitz O, Weintraub AY, Eisenberg VH, Seidman DS, Goldenberg M, Soriano D. Pelvic inflammatory disease in women with endometriosis is more severe than in those without. *Aust N Z J Obstet Gynaecol.* 2014;54:162–5.
35. Sauer MV, Paulson RJ. Pelvic abscess complicating transcervical embryo transfer. *Am J Obstet Gynecol.* 1992;166:148–9.
36. Friedler S, Ben-Shachar I, Abramov Y, et al. Ruptured tubo-ovarian abscess complicating transcervical cryopreserved embryo transfer. *Fertil Steril.* 1996;65:1065–6.
37. Ng SC, Edirisinghe WR, Sathanathan AH, Ratnam SS. Bacterial infection of human oocytes during *in vitro* fertilization. *Int J Fertil.* 1987;32:298–301.
38. Giri SN, Emau P, Cullor JS, et al. Effect of endotoxin on circulating levels of eicosanoids, progesterone, cortisol, glucose and lactic acid, and abortion in pregnant cows. *Vet Microbiol.* 1990;21:211–31.
39. Ben-Rafael Z, Orvieto R. Cytokine involvement in reproduction. *Fertil Steril.* 1992;58:1093–9.
40. Tartakovsky B, Ben-Yair E. Cytokines modulate preimplantation development and pregnancy. *Dev Biol.* 1991;146:345–52.
41. Romero R, Espinoza J, Mazor M. Can endometrial infection/inflammation explain implantation failure, spontaneous abortion, and preterm birth after *in vitro* fertilization? *Fertil Steril.* 2004;82:799–804.
42. Saper CB, Breder CD. Endogenous pyrogens in the CNS: Role in the febrile response. *Prog Brain Res.* 1992;93:419–28.
43. Hanson DF. Fever and the immune response. The effects of physiological temperatures on primary murine splenic T-cell responses *in vitro*. *J Immunol.* 1993;151:436–48.
44. Kitano Y, Okada N. Organization and disorganization of actin filament in human epidermal keratinocyte: Heat, shock treatment and recovery process. *Cell Tissue Res.* 1990;261:269–74.
45. Milunski A, Ulcickas M, Rothman KJ, et al. Maternal heat exposure and neural tube defects. *JAMA.* 1992;268:882–5.
46. Edwards MJ, Mulley R, Ring S, Wanner RA. Mitotic cell death and delay of mitotic activity in guinea-pig embryos following brief maternal hyperthermia. *J Embryol Exp Morphol.* 1974;32: 593–602.
47. Hendricks AG, Stone GW, Hendrickson RV, Matayoshi K. Teratogenic effects of hyperthermia in the bonnet monkey (*Macaca radiata*). *Teratology.* 1979;19:177–82.
48. Russell JB, DeCherney AH, Hobbins JC. A new transvaginal probe and biopsy guide for oocyte retrieval. *Fertil Steril.* 1987;47:350–2.
49. Meldrum DR. Antibiotics for vaginal oocyte aspiration. *J In Vitro Fert Embryo Transf.* 1989;6:1–2.
50. Tsai YC, Lin MY, Chen SH, et al. Vaginal disinfection with povidone iodine immediately before oocyte retrieval is effective in preventing pelvic abscess formation without compromising the outcome of IVF-ET. *J Assist Reprod Genet.* 2005;22:173–5.
51. Borlum KG, Maiggard S. Transvaginal oocyte aspiration and pelvic infection. *Lancet.* 1989;2:53.
52. Van Os HC, Roozenburg BJ, Janssen-Caspers HAB, et al. Vaginal disinfection with povidone iodine and the outcome of *in vitro* fertilization. *Hum Reprod.* 1992;7:349–50.
53. Peters AJ, Hecht B, Durinzi K, et al. Salpingitis or oophoritis: What causes fever following oocyte aspiration and embryo transfer? *Obstet Gynecol.* 1993;81:876–7.
54. Wren M, Parson J. Ultrasound directed follicle aspiration in IVF. In: *Recent Advances in the Management of Infertility.* Chen C, Tan SL, Cheng WC (eds). New York, NY: McGraw-Hill, 1989, pp. 105–81.
55. Ashkenazi J, Ben-David M, Feldberg D, et al. Abdominal complications following ultrasonically-guided percutaneous transvesical collection of oocytes for *in vitro* fertilization. *J In Vitro Fert Embryo Transf.* 1987;4:316–8.
56. Egbase PE, Udo EE, Al-Shharhan M, Grudzinskas JG. Prophylactic antibiotics and endocervical microbial inoculation of the endometrium at embryo transfer. *Lancet.* 1999;354:651–2.
57. Peikrishvili R, Evrard B, Pouly JL, Janny L. Prophylactic antibiotic therapy (amoxicillin + clavulanic acid) before embryo transfer for IVF is useless. Results of a randomized study. *J Gynecol Obstet Biol Reprod.* 2004;33:713–9.
58. Caspi B, Zalel Y, Or Y, et al. Sonographically-guided aspiration: An alternative therapy for tubo-ovarian abscess. *Ultrasound Obstet Gynecol.* 1996;7:439–42.

59. To J, Aldape D, Frost A, Goldberg GL, Levie M, Chudnoff S. Image-guided drainage versus antibiotic only treatment of pelvic abscesses: Short-term and long-term outcomes. *Fertil Steril.* 2014;102:1155–9.
60. Centers for Disease Control and Prevention. Ectopic pregnancy—United States, 1990–1992. *MMWR Recomm Rep.* 1995;1:46.
61. Berg CJ, Atrash HR, Koonin LM, Tucker M. Pregnancy-related mortality in the United States, 1987–1990. *Obstet Gynecol.* 1996;88:161.
62. Steptoe P, Edwards R. Reimplantation of a human embryo with subsequent tubal pregnancy. *Lancet.* 1976;1:1830–2.
63. Azem F, Yaron Y, Botchan A, et al. Ectopic pregnancy after *in vitro* fertilization-embryo transfer (IVF/ET): The possible role of the ET technique. *J Assist Reprod Genet.* 1993;10:302–4.
64. Zouves C, Erenus M, Gomel V. Tubal ectopic pregnancy after *in vitro* fertilization and embryo transfer: A role for proximal occlusion or salpingectomy after failed distal tubal surgery. *Fertil Steril.* 1991;56:691–5.
65. Society for Assisted Reproductive Technology; American Society for Reproductive Medicine. Assisted reproductive technology in the United States: 2001 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology registry. *Fertil Steril.* 2007;87(6):1253–66.
66. Perkins KM, Boulet SL, Kissin DM, Jamieson DJ, National ART Surveillance (NASS) Group. Risk of ectopic pregnancy associated with assisted reproductive technology in the United States, 2001–2011. *Obstet Gynecol.* 2015;125:70–8.
67. Martinez F, Trounson A. An analysis of factors associated with ectopic pregnancy in a human *in vitro* fertilization program. *Fertil Steril.* 1986;45:79–87.
68. Karande VC, Flood JT, Heard N, et al. Analysis of ectopic pregnancies resulting from *in vitro* fertilization and embryo transfer. *Hum Reprod.* 1991;6:446–9.
69. Verhulst G, Camus M, Bollen N, et al. Analysis of the risk factors with regard to the occurrence of ectopic pregnancy after medically assisted procreation. *Hum Reprod.* 1993;8:1284–7.
70. Herman A, Ron-El R, Golan A, et al. The role of tubal pathology and other parameters in ectopic pregnancies occurring in *in vitro* fertilization and embryo transfer. *Fertil Steril.* 1990;54:864–8.
71. Correy JF, Watkins RA, Bradfield GF, et al. Spontaneous pregnancies and pregnancies as a result of treatment on an *in vitro* fertilization program terminating in ectopic pregnancies or spontaneous abortions. *Fertil Steril.* 1988;50:85–8.
72. Pygriots E, Sultan KM, Neal GS, et al. Ectopic pregnancies after *in vitro* fertilization and embryo transfer. *J Assist Reprod Genet.* 1994;11:80–3.
73. Cohen J, Mayaux MJ, Guihard-Moscato ML. Pregnancy outcomes after *in vitro* fertilization. A collaborative study on 2342 pregnancies. *Ann NY Acad Sci.* 1988;54:1–6.
74. Marcus SF, Brinsden PR. Analysis of the incidence and risk factors associated with ectopic pregnancy following *in-vitro* fertilization and embryo transfer. *Hum Reprod.* 1995;10:199–203.
75. Acharya KS, Acharya CR, Provost MP, Yeh JS, Steward RG, Eaton JL, Muasher SJ. Ectopic pregnancy rate increases with the number of retrieved oocytes in autologous *in vitro* fertilization with non-tubal infertility but not donor/recipient cycles: An analysis of 109,140 clinical pregnancies from the society for Assisted Reproductive Technology registry. *Fertil Steril.* 2015;104:873–8.
76. Ankum WM, Mol BW, Van der V, et al. Risk factors for ectopic pregnancy: A meta-analysis. *Fertil Steril.* 1996;66:513–6.
77. Lesny P, Killick SR, Robinson J, Maguiness SD. Transcervical embryo transfer as a risk factor for ectopic pregnancy. *Fertil Steril.* 1999;72:305–9.
78. Clayton HB, Schieve LA, Peterson HB, et al. Ectopic pregnancy risk with assisted reproductive technology procedures. *Obstet Gynecol.* 2006;107:595–604.
79. Londra L, Moreau C, Strobino D, Garcia J, Zacur H, Zhao Y. Ectopic pregnancy after *in vitro* fertilization: Differences between fresh and frozen-thawed cycles. *Fertil Steril.* 2015;104:110–8.
80. Fang C, Huang R, Wei LN, Jia L. Frozen-thawed day 5 blastocyst transfer is associated with a lower risk of ectopic pregnancy than day 3 transfer and fresh transfer. *Fertil Steril.* 2015;103:655–61.
81. Zeng MF, Li LM. Frozen blastocyst transfer reduces incidence of ectopic pregnancy compared with fresh blastocyst transfer: A meta-analysis. *Gynecol Endocrinol.* 2019;35(2):93–9.
82. Liu H, Zhang J, Wang B, Kuang Y. Effect of endometrial thickness on ectopic pregnancy in frozen embryo transfer cycles: An analysis including 17,244 pregnancy cycles. *Fertil Steril.* 2020;113(1):131–9.
83. Russell JB. The etiology of ectopic pregnancy. *Clin Obstet Gynecol.* 1987;30:181–90.
84. Schoolcraft WB, Surrey ES, Gardner DK. Embryo transfer: Techniques and variables affecting success. *Fertil Steril.* 2001;76:863–70.
85. Yovich JL, Turner SR, Murphy AJ. Embryo transfer technique as a cause of ectopic pregnancies in *in vitro* fertilization. *Fertil Steril.* 1985;44:318–21.
86. Friedman BE, Lathi RB, Henne MB, Fisher SL, Milki AA. The effect of air bubble position after blastocyst transfer on pregnancy rates in IVF cycles. *Fertil Steril.* 2011;95:944–7.
87. Knutzen UK, Sotto-Albors CE, Fuller D, et al. Mock embryo transfer (MET) in early luteal phase, the cycle prior to *in vitro* fertilization and embryo transfer (IVF/ET). *Proceedings of the 45th Annual Meeting of the American Fertility Society.* San Francisco, CA, November 13–6, 1989. American Fertility Society, Program Supplement, pS152: 299.
88. Lesny P, Killick SR, Tetlow RL, et al. Embryo transfer—Can we learn anything new from the observation of junctional zone contraction? *Hum Reprod.* 1998;13:1540–6.
89. Job-Spira N, Coste J, Boue J, et al. Chromosomal abnormalities and ectopic pregnancy? New directions for aetiological research. *Hum Reprod.* 1996;11:239–43.
90. Refaat B, Dalton E, Ledger WL. Ectopic pregnancy secondary to *in vitro* fertilisation-embryo transfer: Pathogenic mechanisms and management strategies. *Reprod Biol Endocrinol.* 2015;13:30.
91. Svare J, Norup P, Grove Thomsen S, et al. Heterotopic pregnancies after *in vitro* fertilization and embryo transfer—A Danish survey. *Hum Reprod.* 1993;8:116–8.
92. Tucker M, Smith D, Pike I, et al. Ectopic pregnancy following *in vitro* fertilization and embryo transfer. *Lancet.* 1981;2:1278.
93. Ben-Rafael Z, Mashiach S, Dor J, et al. Treatment independent pregnancy after *in vitro* fertilization and embryo transfer trial. *Fertil Steril.* 1986;45:564–7.
94. Fernandez H, Coste J, Job-Spira N. Controlled ovarian hyperstimulation as a risk factor for ectopic pregnancy. *Obstet Gynecol.* 1991;78:656–9.
95. Jwa SC, Seto S, Takamura M, Kuwahara A, Kajihara T, Ishihara O. Ovarian stimulation increases the risk of ectopic pregnancy for fresh embryo transfers: An Analysis of 68,851 clinical pregnancies from the Japanese Assisted Reproductive Technology registry. *Fertil Steril.* 2020;114(6):1198–206.
96. Fang T, Chen M, Yu W, Ma T, Su Z, Chan DYL, Zhao M, Zheng Q, Wang W. The predictive value of endometrial thickness in 3117 fresh IVF/ICSI cycles for ectopic pregnancy. *J Gynecol Obstet Hum Reprod.* 2021;50(8):102072.
97. Chang HJ, Suh CS. Ectopic pregnancy after assisted reproductive technology: What are the risk factors? *Curr Opin Obstet Gynecol.* 2010;22:202–7.
98. Ben-Rafael Z, Carp HJ, Mashiach S, et al. The clinical features and incidence of concurrent intra and extra uterine pregnancies. *Acta Eur Fertil.* 1985;16:199–202.
99. Dimitry ES, Subak-Sharpe R, Mills M, et al. Nine cases of heterotopic pregnancies in 4 years of *in vitro* fertilization. *Fertil Steril.* 1990;53:107–10.

100. Tal J, Hadad S, Gordon M, et al. Heterotopic pregnancy after ovulation induction and assisted reproduction technologies: A literature review from 1971 to 1993. *Fertil Steril.* 1996;66:1–12.
101. Goldman GA, Fisch B, Ovadia J, Tadir Y. Heterotopic pregnancy after assisted reproductive technologies. *Obstet Gynecol Surv.* 1992;47:217–21.
102. Molloy D, Deambrosio W, Keeping D, et al. Multiplesited (heterotopic) pregnancy after *in vitro* fertilization and gamete intrafallopian transfer. *Fertil Steril.* 1990;53:1068–71.
103. Li HP, Balmaceda JP, Zouves C, et al. Heterotopic pregnancy associated with gamete intra-fallopian transfer. *Hum Reprod.* 1992;7:131–5.
104. Tummon IS, Whitmore NA, Daniel SAJ, et al. Transferring more embryos increases risk of heterotopic pregnancy. *Fertil Steril.* 1964;61:1065–7.
105. Rizk B, Tan SL, Morcos S, et al. Heterotopic pregnancies after *in vitro* fertilization and embryo transfer. *Am J Obstet Gynecol.* 1991;164:161–4.
106. Rojanski N, Schenker JG. Heterotopic pregnancy and assisted reproduction—An update. *J Assist Reprod Genet.* 1996;13:594–601.
107. Maleki A, Khalid N, Patel CR, El-Mahdi E. The rising incidence of heterotopic pregnancy: Current perspectives and associations with *in-vitro* fertilization. *Eur J Obstet Gynecol Reprod Biol.* 2021;266:138–44.
108. Oriol B, Barrio A, Pacheco A, et al. Systemic methotrexate to treat ectopic pregnancy does not affect ovarian reserve. *Fertil Steril.* 2008;90:1579–82.
109. Orvieto R, Kruchkovich J, Zohav E, et al. Does methotrexate treatment for ectopic pregnancy influence the patient's performance during a subsequent *in vitro* fertilization/embryo transfer cycle? *Fertil Steril.* 2007;88:1685–6.
110. Wiser A, Gilbert A, Nahum R, et al. Effects of treatment of ectopic pregnancy with methotrexate or salpingectomy in the subsequent IVF cycle. *Reprod Biomed Online.* 2013;26:449–53.
111. Rock JA, Damario MA. Ectopic pregnancy. In: *Te Linde's Operative Gynecology.* 8th edn. Rock JA, Thompson JD (eds). Philadelphia, PA: Lippincott-Raven, 1997, pp. 501–27.
112. Yao M, Tulandi T. Current status of surgical and non-surgical management of ectopic pregnancy. *Fertil Steril.* 1997;67:421–33.
113. Bhandari H, Agrawal R, Weissman A, Shoham G, Leong M, Shoham Z. Minimizing the risk of infection and bleeding at trans-vaginal ultrasound-guided ovum pick-up: Results of a prospective web-based world-wide survey. *J Obstet Gynaecol India.* 2015;65(6):389–95.

EGG AND EMBRYO DONATION

Mark V. Sauer and Haley G. Genovese

Introduction

Human egg (oocyte) and embryo donation was first introduced in 1983 and has evolved over the past four decades into a relatively common procedure that addresses a variety of reproductive disorders. This method has provided key insights into the physiology and pathophysiology of reproduction and, like other assisted reproduction technologies (ARTs), has engendered its share of controversy. Furthermore, techniques introduced by egg donation, such as schemes for adequate hormonal preparation of the uterus for synchronizing embryos with a receptive endometrium, have been successfully applied to other fertility therapies, including the management of patients with cryopreserved embryos for transfer and those requiring *in vitro* maturation of immature oocytes.

Although use of oocyte and embryo donation in cattle was common during the 1970s to improve animals' reproductive efficiency [1], initial work on oocyte donation in humans did not begin until 1983, when researchers at the University of California, Los Angeles fertilized an oocyte *in vivo* after the artificial insemination of a human donor and then transferred the recovered embryo into a synchronized recipient [2]. A total of 14 insemination cycles resulted in two ongoing pregnancies [3]. In 1984, the first delivery of a healthy male infant was reported [4].

The popularity of egg and embryo donation has grown significantly since this technique was first developed [5]. In the United States, more than 22,000 procedures involving fresh or frozen embryos procured through oocyte donation or embryo donation were reported to the Centers for Disease Control and Prevention in 2018 [5]. Patients who undergo these cycles are largely those who have deliberately delayed childbearing to older ages, to pursue other goals. Unfortunately, there is a natural decline in fertility associated with advancing age, and many healthy women later experience difficulties because of normal aging.

Indications for egg and embryo donation

The indications for egg and embryo donation have expanded since its inception. Originally envisioned as a fertility treatment for women with premature ovarian insufficiency (POI) [6], today women with many other reproductive disorders are considered prime candidates for therapy ([Table 71.1](#)).

Non-iatrogenic POI, defined as women <40 years old with persistent amenorrhea and elevated gonadotropins, affects approximately 1% of the female population [7]. The majority of cases are idiopathic, but about 20%–30% are suspected of being autoimmune in nature or the result of concomitant glandular autoimmune disease [8]. Thus, it is important to ensure that clinical or subclinical failure of the thyroid, parathyroid, and adrenal glands does not coexist, as well as diabetes mellitus and myasthenia gravis. Any of these conditions may adversely affect pregnancy

outcome as well as impact upon the general health and well-being of the patient. If POI occurs at <30 years old, a karyotype should also be requested to ascertain the presence of Y-chromosome mosaicism. Patients discovered to be mosaic are at risk of gonadal tumours and require extirpation of the abnormal gonad [9]. In addition, a bone density evaluation is helpful to identify patients with osteopenia or osteoporosis, which may be present despite hormone replacement therapy [7]. Turner syndrome is the most common gonadal dysgenesis in women, with a prevalence of 1 in 2500 live-born females. Both spontaneous puberty and spontaneous pregnancy are relatively rare in these patients, occurring in less than 5% of affected individuals [10]. Other rare conditions associated with POI include congenital thymic aplasia (e.g. DiGeorge syndrome) [11], galactosemia [11], Swyer syndrome (e.g. 46,XY pure gonadal dysgenesis) [12], along with a variety of rare mutations in genes critical for ovarian development or function [12].

A common cause of POI that has been picked up more frequently with the increased use of genetic screening is fragile X pre-mutation. Pre-mutation of more than 55 but less than 200 CGG repeats is not associated with fragile X syndrome; however, this has been shown to be associated with POI. Approximately 20%–30% of women who have this fragile X pre-mutation will suffer from POI [13].

Chemotherapy and radiation treatments for cancer may also lead to POI. Gonadotoxicity is age and dose dependent, though it can be difficult to predict a given patient's susceptibility to loss of ovarian function [14]. There is currently ongoing research into identifying genetic markers that may signal a relative increased or decreased risk of POI with chemotherapy [14]. Removal of the ovaries is often required for treatment of malignancies, but surgical castration more commonly results from non-cancerous conditions, including infection, torsion, or overly aggressive removal of intra-ovarian lesions (e.g. cystic teratomas and endometriomas).

Repetitive failure at IVF is common when a poor ovarian response to gonadotropins occurs. Occasionally, patients are identified as poor candidates for IVF treatment prior to initiating care, thus sparing them the expense and psychological distress of multiple failed cycles. The first consideration is the age of the patient. It has long been known that natural fertility decreases with age, as does the success rate with IVF ([Figure 71.1](#)) [15]. Many IVF centres have a maximum age limit beyond which they will not perform IVF without oocyte donation. Women of advanced reproductive age have far greater success with donated oocytes [16]. Ovarian reserve is evaluated with serum follicle-stimulating hormone (FSH) levels on day 2, 3, or 4 of the menstrual cycle [17]. Values >15 mIU/mL are prognostic for a greatly reduced IVF success rate. Another useful serum marker to aid in interpretation of FSH values is basal oestradiol level [17]. Values >60–80 pg/mL suggest diminished ovarian reserve. It is important that each laboratory determines the threshold values that are useful for their programme.

TABLE 71.1 Indications for Oocyte Donation

Premature ovarian failure
Gonadal dysgenesis
Repeat <i>in vitro</i> fertilization failure
Natural menopause
Inheritable disorders
Same-sex couples

Anti-Müllerian hormone (AMH) is produced in the granulosa cells from preantral and small antral follicles, and serum levels are measurable and reflective of ovarian reserve. Higher levels of AMH are associated with greater numbers of retrieved oocytes in women undergoing IVF, while low levels appear to be reliable markers for diminished ovarian reserve [17]. Thus, AMH testing may identify women at risk for either extreme (hypo- or hyper-) in ovarian responsiveness. It has been proposed that candidates undergoing evaluation to donate oocytes have an AMH >1.5 ng/mL to ensure adequate ovarian reserve prior to undergoing stimulation [18]. Other tests such as the clomiphene challenge test are extant to assess ovarian reserve, but are more cumbersome than day-3 serum FSH and oestradiol and are becoming obsolete [17]. A low serum inhibin B in the early follicular phase also suggests diminished ovarian reserve, as this hormone is a direct measurement of the follicular pool [17].

Antral follicle count is another marker of ovarian reserve. Antral follicles are the follicles measuring between 2 and 10 mm during the early follicular phase. A low antral follicle count defined as <10 in total is associated with poor ovarian reserve. A low antral follicle count is suggestive of poor ovarian reserve, but

is a less accurate predictor of oocyte yield, quality, IVF success, and pregnancy outcome [19]. For this reason, AMH level may be a superior assessment of ovarian reserve [20] and can additionally lend some predictive power to identifying patients with either poor or excessive response to stimulation [18].

In certain cases, ovarian stimulation is adequate, but fertilization rates are poor and often oocyte quality is marginal. Intracytoplasmic sperm injection (ICSI) is known to improve fertilization rates and IVF outcomes in couples with male factor infertility, but if fertilization failure is persistent, then oocyte donation is reasonable [21]. Similarly, successful fertilization may be present, but implantation still might not occur. Assisted hatching may be helpful in these cases. Both ICSI and assisted hatching are discussed in detail in other chapters, but the belief is that recurrent implantation failure is often secondary to poor gametes and may be overcome by oocyte donation. Less clear is the patient with recurrent pregnancy loss, although at least one report suggests that oocyte donation is effective in these cases as well [22]. Finally, in rare instances, IVF failure may be due to ovaries that are inaccessible to either transvaginal or laparoscopic retrieval, and oocytes can be provided only through donation.

Oocyte donation to treat infertility in women with physiological menopause is an established and very effective method to achieve pregnancy in patients who have reached the end of their reproductive years [23]. When this treatment was initially introduced, it was quite controversial; the Ethics Committee of the American Society for Reproductive Medicine (ASRM) stated that because of the physical and psychological risks involved (to both mother and child), oocyte donation in postmenopausal women should be discouraged [16]. However, more recent recommendations state that physicians should primarily consider the patient's health status to determine candidacy for pregnancy [16]. Embryo

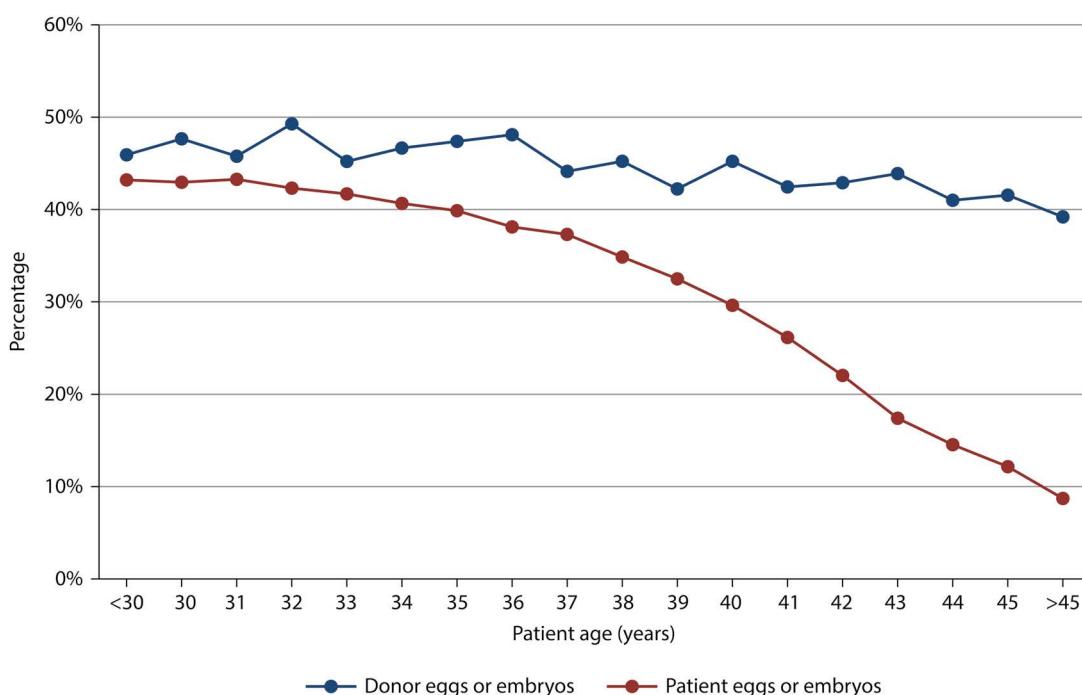


FIGURE 71.1 Percentage of embryo transfers that resulted in live-birth delivery, by patient age and egg or embryo source, United States, 2019. (2019 National Summary Report on ART success rates, CDC: <https://www.cdc.gov/art/artdata/index.html#reports?>)

transfer is discouraged in patients with underlying health conditions that might significantly increase obstetrical risks, as well as those over age 55, due to concerns regarding longevity and increased risks of pregnancy with advanced age. This shift was brought about in part due to reassuring data on pregnancy outcomes in appropriately screened women [24]. However, providers and patients should be aware that pregnancies conceived via oocyte donation are at increased risk of certain obstetrical and neonatal complications, including pre-eclampsia, preterm delivery, and low birthweight [25].

Less controversial is the use of egg donation for inheritable conditions such as X-linked or autosomal traits and chromosomal translocations. However, with progress in pre-implantation diagnosis, this reason for choosing egg donation has decreased [26].

Individuals in the LGBTQ community commonly seek fertility care to build their families. For such patients, the use of oocyte donation and/or gestational carriers may be necessary, and use of these services has continued to increase [27]. While acceptance of gay and lesbian individuals who are seeking fertility care has improved significantly, these patients may still face discrimination and decreased access to care [28]. The Ethics Committee of the ASRM has issued guidelines that call for a non-discriminatory policy in treating all patients requesting fertility assistance [28]. Furthermore, transgender patients requesting fertility treatments may face even greater barriers to care [29]. This is particularly detrimental, as providers should have the opportunity to counsel transgender individuals on fertility preservation options prior to these patients undergoing gender-affirming treatments [29]. The Committee emphasizes the importance of providing culturally competent care and offering treatment to all patients who require it, regardless of gender identity.

Recipient screening

In addition to a complete history and physical examination, the suggested medical screening for recipients is shown in Table 71.2. Most of the tests are requisite standards for expectant mothers and IVF candidates. Patients of advanced maternal age are at higher risk for certain conditions such as diabetes mellitus, hypertension, and heart disease and therefore require additional testing focused on these disorders. Other recipients may warrant more comprehensive evaluations, such as a karyotype and autoimmune screen in patients with POI, or screening for anomalies of the aorta and urological system in patients with gonadal dysgenesis.

Psychological screening of recipient couples is also recommended. The stress that infertility places on relationships is well known [30]. Furthermore, with respect to oocyte donation, the resulting child will not be genetically related to the mother. Most couples reconcile themselves to this, and research has shown that the desire to be parents is more important for positive parenting than a genetic link with the child [31]. However, it remains important to address any grief, anxiety, and depression directly with the couple prior to proceeding. The role of the mental healthcare professional is usually one of support and guidance for the couple struggling with these issues. Occasionally, a couple is found to have greatly disparate ideas of what the pregnancy will accomplish. A pregnancy conceived merely to salvage a marriage or relationship is best deferred until the couple resolves their differences.

Endometriosis is known to affect oocyte quantity and quality. In patients with endometriosis who are undergoing IVF with a

TABLE 71.2 Suggested Medical Screening of Oocyte Recipient(s)

Oocyte Recipient	Male Partner
Complete blood count with platelets	Blood Rh and type
Blood Rh and type	Hepatitis screen
Serum electrolytes, liver, and kidney function	VDRL
Sensitive TSH	HIV-1, HTLV-1
Rubella and hepatitis screen	Semen analysis and culture
VDRL	
HIV-1, HTLV-1	
Urinalysis and culture	
Cervical cultures for gonorrhoea and chlamydia	
Pap smear	
Transvaginal ultrasound	
Uterine cavity evaluation (sonohysterogram, diagnostic hysteroscopy, or hysterosalpingogram)	
Electrocardiogram	
Chest X-ray	
Mammogram	
Haemoglobin A1C	

Abbreviations: HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; TSH, thyroid-stimulating hormone; VDRL, Venereal Disease Research Laboratory.

donor oocyte from an unaffected individual, implantation rates are similar to patients without endometriosis [32]. Conversely, patients without endometriosis who undergo treatment with oocytes donated from an individual who is diagnosed with endometriosis have lower implantation rates [32].

A hydrosalpinx is also known to be deleterious and has been shown to result in lower implantation and pregnancy rates [33]. Surgical treatment with laparoscopic salpingectomy to remove the damaged tube is recommended [33]. Salpingostomy is an alternative procedure for women desiring natural conception, but it may result in greater risk of ectopic pregnancy. Prior to beginning an IVF cycle, recipients should have a normal uterine cavity free of adhesions, space-occupying lesions, and pathology. This is best assessed by a pre-cycle sonohysterogram or diagnostic hysteroscopy.

Endometrial hypoproliferation occurs when the endometrial lining does not achieve adequate thickness; various cut-offs for this number have been used, but commonly hypoproliferation is defined as endometrial thickness (EMT) <7 mm [34]. While a thinner EMT of ≤6 mm is not prohibitive to conception, it is associated with a slightly increased miscarriage rate as well as several obstetric complications, including intrauterine growth restriction and preterm delivery [35]. Fortunately, endometrial hypoproliferation occurs rather infrequently—one meta-analysis found EMT <7 mm in just 2.4% of patients [34]. A Cochrane review failed to confirm any one protocol for optimizing endometrial preparation with regards to pregnancy rate in a retrospective analysis of 22 randomized controlled clinical trials [36].

Oocyte donor recruitment

Perhaps the greatest obstacle to performing oocyte donation is the recruitment of suitable donors [37]. Historically, donor eggs were obtained from women undergoing IVF with “excess

oocytes." Many of these patients had ovarian abnormalities underlying their own infertility, making them imperfect donors. Furthermore, with the advent of increasingly successful embryo cryopreservation and the use of "softer" stimulation protocols, "extra oocytes" have become scarce. Known designated donors are another option. Typically, a family member (e.g. sister or niece) or very close friend is selected. The final sources of donors are women recruited from the general population at large, most often through advertisement.

In today's ART clinic, donor eggs are frequently obtained from "egg banks," which store oocytes from young, healthy donors who were paid to undergo stimulation and oocyte retrieval. This concept has been the source of a long-standing debate as to whether it is ethical to pay oocyte donors for their eggs, and if so, how much. Areas of contention include the selling of body parts and exaggerated incentives that may represent an enticement for a procedure that carries risk and no direct medical benefit to the donor [38]. For this reason, many countries do not permit commercial oocyte donation (e.g. Germany, Italy). Other locales allow only IVF patients with excess oocytes to donate. Australia and the United Kingdom do not allow payment to the donor, except for verified expenses. However, restrictions in certain countries and lack of restrictions in others has paved the way for medical tourism; motivated patients who have the means to travel can and do seek care in countries with fewer regulations. For example, a recent case report described a 65-year-old German woman who travelled to Ukraine and underwent IVF with donor oocytes and donor sperm, resulting in a quadruplet pregnancy [39]. The infants were delivered at 25 weeks' gestation, and unfortunately suffered multiple complications in their neonatal course.

The United States has regulations in place addressing who can be an oocyte donor and multiple guidelines for patients' eligibility to undergo IVF, but no current regulations exist regarding payments to donors. The payments are construed as reimbursement for time, inconvenience, and risks of undergoing stimulation and retrieval [40]. Without payment, it remains doubtful that a country will recruit sufficient donors to meet demand, but the appropriate amount of payment remains hotly debated. ASRM states that it should be "fair," but not be so high that it inordinately influences or entices potential donors [40].

Another area of controversy focuses on anonymity and identity disclosure. Many donors express a strong desire not to be identified by the children. In exchange for anonymity, they willingly forfeit all legal obligations as parents. However, in the age of easy internet searchability and widely accessible commercialized genetic testing, offspring may eventually seek out donors regardless of the donor's wishes. Moreover, some critics believe that similar to adopted children, offspring of egg donation should have the same right to ultimately identify their genetic parent [41]. As a result, the UK now mandates that donor identity be revealed to a child resulting from egg donation once he or she reaches the age of 18 years. In the United States, there is little historic precedent for such a change in public policy, but should legislation ever be enacted, a deleterious effect on donor recruitment can be expected [42].

An alternative to cycle-synced egg donation is the "egg bank" model in which donor oocytes are cryopreserved and stored for future use by patients who require them. The number of egg banks in the United States has continued to grow alongside the demand for donor oocytes [43]. For recipients, the appeal of using an egg bank is great. Egg banks remove wait times, allow for longer quarantine periods permitting a longer infectious disease

screen, and, in many cases, offer more options in terms of donors [43]. For clinicians, egg banks are an excellent treatment option for patients who would be otherwise unable to achieve pregnancy with their own oocytes. Pregnancy and live birth rates for women utilizing donor oocytes are higher than those of women who are utilizing their own oocytes, even among young patients [44]. However, egg banks are associated with several potential areas of concern. While most studies indicate that fresh oocytes yield higher numbers of fertilized zygotes and useable embryos [45], it remains unclear whether cryopreserved donor oocytes obtained from an egg bank achieve the same pregnancy or live birth rates as fresh donor oocytes [43, 46]. A recent study of 33,863 donor oocyte recipients undergoing fresh embryo transfer found that fresh donor oocytes were associated with higher pregnancy and live birth rates than cryopreserved-thawed donor oocytes [47]. However, earlier work compared ongoing pregnancy rates and found that fresh oocytes have no superiority over vitrified oocytes [48]. This approach is also very costly for recipients, who often pay several thousand US dollars per oocyte [43]. Finally, it is worth noting that egg banks introduce additional ethical considerations, and physicians must account for the wellbeing of the donor, the recipient, and the potential child that might result from treatment. There is currently minimal emphasis on regulating the storage and distribution of oocytes once they are in the egg bank [38]. In the future, it may be of benefit to have a centralized governing body to manage donated gametes.

Oocyte donor screening

Oocyte donors need to provide full and comprehensive informed consent. The risks of participating in oocyte donation are few and are basically no different from those of standard IVF. Controlled ovarian stimulation entails both known and theoretical risks. The risk of severe ovarian hyperstimulation syndrome (OHSS) is reported in approximately 1% of cases, although donors may be at less risk of severe OHSS compared with patients undergoing IVF, since pregnancy does not occur in the donor and moderate cases of OHSS are therefore not exacerbated [49]. Using gonadotropin-releasing hormone (GnRH) agonist to trigger final oocyte maturation has been shown to significantly reduce the occurrence of OHSS compared to using human chorionic gonadotropin and represents a valid alternative for egg donors to further reduce morbidity [50]. In addition to a complete medical history and physical examination, the suggested medical screening of oocyte donors is shown in Table 71.3. Of utmost importance is the screening for infectious diseases. Unlike sperm, which are amenable to cryopreservation, oocytes have traditionally not been

TABLE 71.3 Suggested Medical Screening of Oocyte Donors

Complete blood count with platelets
Blood type
Hepatitis screen
VDRL, HIV-1, HIV-2, HIV group O antibody
West Nile Virus NAAT
Cervical cultures for gonorrhoea and chlamydia
Pap smear
Transvaginal ultrasound of pelvis
Appropriate genetic tests

Abbreviations: HIV, human immunodeficiency virus; CMV, cytomegalovirus; VDRL, Venereal Disease Research Laboratory.

frozen for subsequent use. In sperm donation, cryopreservation allows a quarantine period and follow-up testing for infectious diseases. With respect to current practice, egg cryopreservation has not been universally adapted to oocyte donors. Transvaginal ultrasound examination is performed to detect pelvic pathology and determine ovarian morphology.

It is recommended that oocyte donors in the United States be between 21 and 34 years old, as this is thought to minimize psychological harm to donors and maximize pregnancy rates for recipients [51]. Within this age range, younger donors are not superior to older donors. A retrospective cohort analysis found that donors ≤25 years of age had a similar number of oocytes retrieved, and similar blastulation rates as well as euploid blastocyst rates compared with donors in older age groups (up to age 34) [51]. In circumstances in which the donor is ≥35 years of age, the recipient should be counselled regarding potential genetic risks and the expectation for reduced pregnancy rates [52]. The prior fertility history of the donor does not appear to affect pregnancy outcomes [49].

The concept of a “proven” donor is a popular myth, and lacks evidence-based support. Other factors such as obesity and smoking are also known to influence ART outcomes. Ideally, donors should have a body mass index ≤28 kg/m², as one retrospective study of 2722 oocyte donor cycles found no difference in clinical pregnancy or live birth rates up that point [53]. Donors should also be non-smokers, as it has been well demonstrated in the literature that smoking leads to poorer ovarian response [54].

Psychological evaluation by a licensed mental health practitioner is recommended for anonymous donors and is mandatory for known donors. Screening should focus on their motivation to donate, as well as their financial status to ensure that their participation is not overly influenced by monetary enticement. An assessment of coping skills and lifestyle is important to predict the donor’s ability to participate in a lengthy and complicated process.

Occasionally, a history of psychiatric illness or drug and/or alcohol use in the donor or her family is elicited. These behaviours may have a genetic aetiology and as such would exclude the potential donor from participation. Genetic screening begins with a detailed history of the potential donor and her family. A sample history form is presented in Table 71.4 [55]. The presence of any of the disorders should exclude her from participating. Selecting donors <35 years old reduces the risk of aneuploidy in the offspring, however exceptions can be made in circumstances such as sister-to sister donation where the benefits of a shared genetic background may balance the known risks (which can be largely discovered by amniocentesis). A donor should not have any major Mendelian disorder. These include cystic fibrosis in whites, a sickle cell anaemia test for blacks, and a complete blood count and mean corpuscular volume followed by haemoglobin electrophoresis in abnormal results for people of Mediterranean and Chinese ancestry to assess the risk of β-thalassemia, and in people of Southeast Asian ancestry for α-thalassemia. Jews of eastern European ancestry should be screened for Tay–Sachs disease, Gaucher disease, mucolipidosis IV, Niemann–Pick disease, Bloom syndrome, familial dysautonomia, Fanconi anaemia, fragile X syndrome, and Canavan disease. A donor should not have any major malformation of complex cause, such as spina bifida or heart malformation. A donor should not carry a known karyotypic abnormality that may result in chromosomally unbalanced gametes. If a donor is a member of a high-risk group, then the donor must be screened for carrier status [56]. It is important to

inform the recipient couples that carrier screening does not identify all individuals who are at risk of a disease, and a negative carrier screen does not guarantee absence of a mutation [57].

Legal consultation is recommended for cases involving directed donation, in which the donor individual is known to the recipient but not in a relationship with that individual. This can clarify the expectations and parental rights of each party and prevent disagreements regarding custody of the embryos or future offspring. Donors should also receive contraception counselling and should utilize contraception while undergoing ovarian stimulation. A recent meta-analysis found that the use of a levonorgestrel intrauterine device or progestin contraceptive pill during ovarian stimulation does not affect donor oocyte yield or pregnancy rates in recipients [18].

Guidelines for gamete and embryo donation have been periodically published and were most recently updated in 2021 to standardize screening policies and to incorporate regulations from the US Food and Drug Administration (FDA) [56]. In recent years, outbreaks of viral infectious diseases have resulted in changes in policy. The Zika outbreak first appeared in the United States in 2016 and was recognized to cause serious birth defects. This resulted in FDA recommendations to screen oocyte donors for possible exposure via a questionnaire. While Zika antibody tests have been developed, there is currently no requirement to screen oocyte donors for this virus via laboratory testing [58]. More recently, the Covid-19 pandemic universally disrupted care and altered protocols in many areas of medicine, including within ART centres. At present, there is no requirement to screen oocyte donors for the Covid-19 virus and there have been no documented cases of Covid-19 transmission via a donor oocyte cycle.

Endometrial stimulation and synchronization

Endometrial preparation of the recipient is modelled on the natural menstrual cycle, using oestrogen and progesterone [16]. There are multiple different protocols for endometrial preparation, which are discussed in greater detail elsewhere. A recent Cochrane review reported that there is insufficient evidence to recommend any specific intervention for endometrial preparation in patients undergoing fresh donor cycles and frozen embryo transfers [36]. If a patient is having regular menstrual cycles, it is also possible to sync the timing of transfer with her natural cycle.

The initial estrogenic phase is most often maintained using either daily oral oestradiol 4–8 mg or transdermal oestrogen 0.2–0.4 mg. The length of estrogenic exposure may vary widely with little apparent clinical effect, again mimicking the variable follicular phase found in natural menstrual cycles [59]. Most programmes prescribe at least 12–14 days of oestrogen before initiating progesterone, but studies report that if it is necessary to prolong this period, perhaps because of a slow stimulation of the oocyte donor, no adverse effects are expected [60].

With the increased popularity of egg banks in recent years, it has become more common to have a donor’s oocytes cryopreserved for later use by a recipient. In such cases, there is obviously no need for synchronization of cycles between the donor and the future recipient. However, if a fresh cycle is undertaken, synchronization of the recipient and donor is relatively easy to accomplish. The recipient begins oestrogen several days prior to beginning ovarian stimulation in the donor to provide approximately 14 days of oestradiol prior to progesterone administration. Ovulating recipients typically receive GnRH agonist for

TABLE 71.4 Genetic Screening Form Given to Oocyte Donors

Pregnancy history: Please list all the times you have been pregnant and the outcomes.

Family ethnic background: Please indicate all relevant information in the following tables. When the requested information is unknown, please say so.

If comments are needed, please make them. Remember that we are interested in your genetic background. If any relevant family member is adopted, please say so.

Relation	Age if Living	Age at Death	Cause of Death		
Grandfather (paternal)					
Grandmother (paternal)					
Grandfather (maternal)					
Grandmother (maternal)					
Father					
Mother					
Brothers					
Sisters					
Family genetic history					
Familial conditions	Self	Mother	Father	Siblings	Comments
High blood pressure					
Heart disease					
Deafness					
Blindness					
Severe arthritis					
Juvenile diabetes					
Alcoholism					
Schizophrenia					
Depression or mania					
Epilepsy					
Alzheimer's disease					
Other (specify)					
Malformations					
Cleft lip or palate					
Heart defect					
Clubfoot					
Spina bifida					
Other (specify)					
Mendelian disorders					
Colour blindness					
Cystic fibrosis					
Haemophilia					
Muscular dystrophy					
Sickle cell anaemia					
Huntington's disease					
Polycystic kidneys					
Glaucoma					
Tay–Sachs disease					
Please take the time to explain any other problems or conditions in your family history that you feel could pertain to the health of future generations.					

Source: From [45], with permission.

downregulation as in standard IVF cycles (e.g. 1 mg leuprolide acetate daily until suppressed, then 0.5 mg daily thereafter) to render them functionally agonadal. Alternatively, ovulating recipients are started on oral oestrogen at the beginning of their menstrual cycle and maintained on oestrogen, and a GnRH antagonist is used to block the LH surge, until the day of the donor's oocyte retrieval when progesterone is begun (Figure 71.2) [61]. The timing of progesterone administration is more stringent.

In fresh synchronized donor oocyte cycles, there is some evidence that clinical pregnancy rates are improved when progesterone is started on the day of or the day after donor oocyte retrieval [36].

In oocyte donors, ovarian stimulation is typically undertaken with injectable gonadotropins (150–225 IU) beginning on cycle day 2 or 3. If necessary, donors can alternatively undergo a random start cycle with no negative impact on ovarian response. An LH suppression protocol, using either a GnRH agonist or GnRH

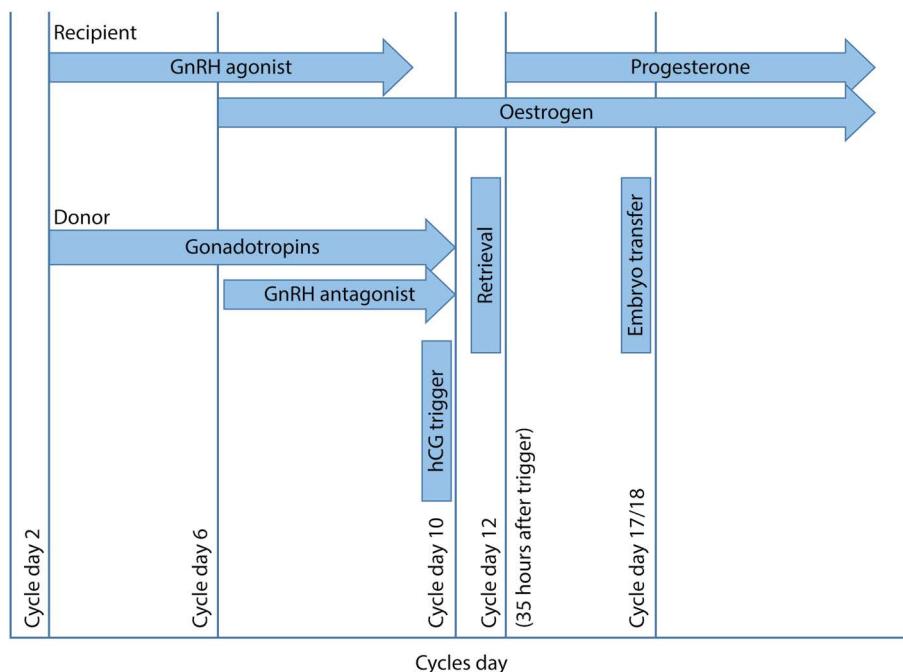


FIGURE 71.2 Schematic representation of cycle synchronization using a GnRH agonist in both donor and recipient. GnRH agonists are used to downregulate the pituitary of recipients with evidence of ovarian activity prior to beginning oral oestradiol. Oral oestradiol is prescribed to the recipient four to five days in advance of the donor starting gonadotropin injections. Progesterone is administered starting the day after hCG injection in the donor, and one day prior to aspirating oocytes. Embryo transfer is performed three days following oocyte retrieval. Serum pregnancy testing occurs 12 days post-transfer. Pregnant patients are maintained on oestradiol and progesterone through to 12 weeks of gestational age. Abbreviations: GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin.

antagonist, is then utilized to prevent the LH surge. A recent meta-analysis compared protocols using GnRH agonist with those using GnRH antagonist and found no difference in ovarian response, but recommended routine utilization of GnRH antagonist protocols in order to best maximize oocyte yield and minimize the risk of OHSS [18]. Patients are then monitored with serial transvaginal ultrasonography and a GnRH agonist trigger is administered at the appropriate time.

There are currently no additional medications or supplements that are recommended for oocyte donors. While prior work has investigated the effect of folic acid supplementation on ovarian stimulation outcomes, results have been mixed and oocyte donors were excluded from the study populations [62, 63].

As practices have moved towards a greater number of frozen embryo transfers in recent years, other protocols such as progestrone primed ovarian stimulation (PPOS) have gained popularity for their decreased cost and ease of using oral medications instead of injections. This protocol involves oral administration of progestin in lieu of GnRH analogues throughout the stimulation cycle to prevent the LH surge [18]. These qualities are highly desirable to potential oocyte donors, and such protocols may become increasingly utilized in the future for OD patients. However, further data regarding pregnancy outcomes in recipients and neonatal outcomes is needed.

The progestrone dose and route of administration varies between different IVF centres. Many groups prefer the transvaginal approach because lower serum concentrations of progestrone are required to achieve target organ effect and pregnancy outcomes appear to be equivalent [64].

Traditionally, progesterone (and oestrogen) administration is discontinued once the placenta has established adequate steroidogenesis to support the pregnancy. Devroey et al. estimated this to occur at seven to nine weeks of gestation [65]. However, recent evidence suggests that supplemental progesterone can be discontinued at the time of positive pregnancy test without compromising pregnancy outcomes [66]. Clinically, we begin weekly monitoring of serum progesterone concentrations 10 weeks after embryo transfer when a serum level of ≥ 30 ng/mL is typically attained. At that point, prescribing exogenous steroids is superfluous.

Clinical and obstetric outcomes

Recipients of donated eggs experience implantation and pregnancy rates similar to those normally seen in young women undergoing IVF. Thus, the ASRM now recommends single embryo transfer (when the age of the donor is < 38 years, and outcomes are otherwise expected to be favourable) to lessen the risk of multiple gestation [67]. It is increasingly common for clinics to utilize pre-implantation genetic testing for aneuploidies (PGT-A) to select better embryos for transfer, since oocytes from even young, healthy donors produce approximately 25% aneuploid blastocysts [51, 68]. While transferring an embryo that has been identified as euploid via trophectoderm biopsy may decrease the time to pregnancy, it does not appear to improve recipients' live birth rates or lower miscarriage rates [69]. There is ongoing debate regarding the utility of PGT-A in this population, especially given that it comes at increased fiscal cost to the patient.

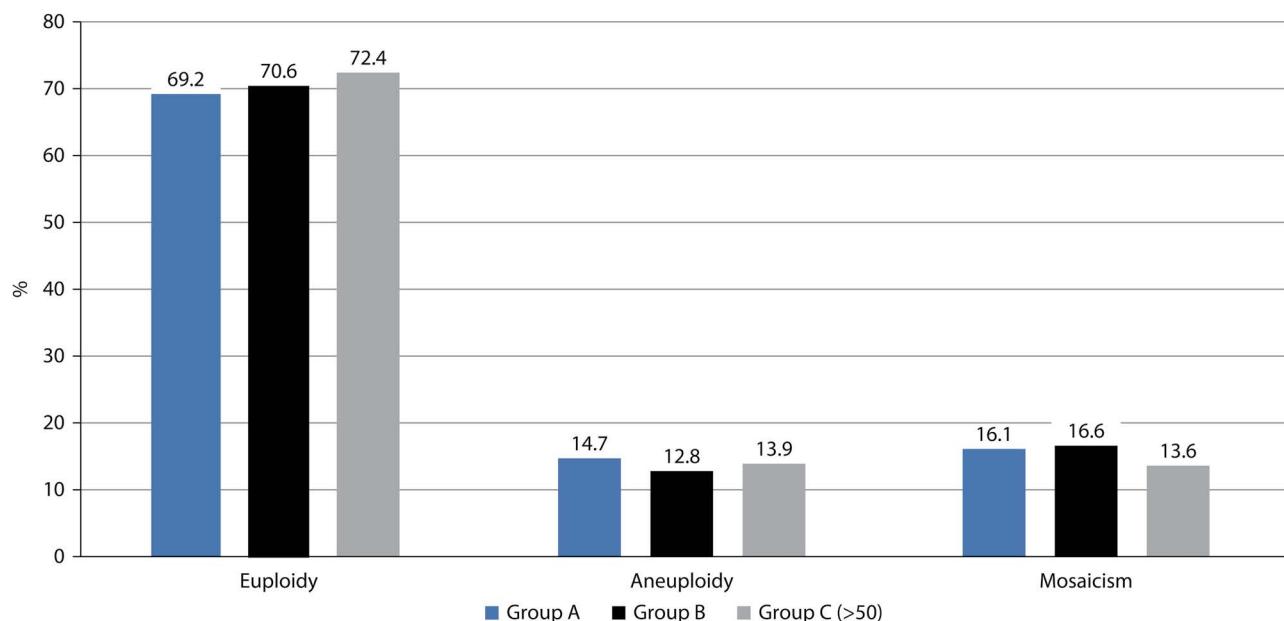


FIGURE 71.3 Ploidy rates for assisted reproduction technology cycles using PGT-A tested embryos from donor eggs, by male partner's age, 2020: paternal age group A (blue) ≤ 39 years ($n = 592$); B (black) 40–49 years ($n = 496$); C ≥ 50 years ($n = 97$). (From [69], with permission.)

In evaluating a couple's fertility potential, attention has historically focused on the female partner's age. Egg donation has been applied to treat infertility in women of advanced reproductive age since 1990 and has soared in popularity because of its ability to reverse the inevitable loss of fertility in women approaching menopause [16]. Patients who are of very advanced maternal age, even into their 50s, continue to have high rates of success with donor oocyte despite reaching the age of physiologic menopause. This suggests that the uterus and endometrium continue to be receptive to pregnancy and have a significantly extended lifespan compared to the ovary [70]. However, there is still some concern that endometrial receptivity declines with advancing maternal age. Yeh et al. published a retrospective cohort evaluation of 27,959 fresh donor IVF (DIVF) cycles comparing implantation, clinical pregnancy, and live birth rates among recipient age groups [71]. They found that all these outcomes were significantly decreased in recipients older than 45 years. Moreover, rates were significantly worse in the >50 years old group compared to the 45–49 years old group [71].

Several groups have evaluated the obstetric outcomes of pregnancies following oocyte donation and concluded that results are favourable [71, 72]. However, these pregnancies are associated with an increased incidence of multiple obstetric complications. Rizzello et al. compared delivery data from 276 pregnancies conceived with oocyte donation to 925 pregnancies conceived via autologous oocytes as well as 24,650 spontaneous conceptions; they noted a higher rate of pregnancy induced hypertension (PIH) compared with spontaneous conceptions (aOR 3.6) and IVF pregnancies (aOR 2.7) [72]. Caesarean section rates were higher in oocyte donation pregnancies compared with spontaneous conceptions (aOR 3.4) and compared with IVF pregnancies (aOR 2.3) [72]. While use of donor oocytes is an independent risk factor for many pregnancy comorbidities, the impact of maternal age appears to be less pronounced in this group of patients compared with those undergoing IVF with autologous oocytes

[73]. In summary, oocyte donation pregnancies should be considered high risk. However, in well-screened patients, the complications are manageable, and parents can reasonably expect healthy children.

Additional factors that may affect DIVF outcomes include male age and racial differences (particularly in the black population). A recent study by McCarter et al. suggests that advanced paternal age (defined as ≥ 45 years old) is associated with decreased pregnancy rates in donor oocyte cycles [74]. It is possible that this effect may be related to increased DNA fragmentation contributing to poorer sperm quality at older ages. However, another large study by Dviri et al. performed PGT-A on more than 3000 embryos derived from oocyte donors and found no impact of paternal age on aneuploidy rates (Figure 71.3) [75]. However, they did report decreased fertilization rates in males ≥ 50 years old compared with younger male partner age.

Race, particularly black race, can lead to poorer reproductive outcomes in DIVF cycles. Oocyte donation has always been associated with high success rates, and more than 50% of embryo transfers in white recipients result in live births. However, Zhou, et al. conducted a large retrospective analysis of 926 oocyte recipients and reported the live birth rate was just 32% among black recipients and was further reduced to 22% in black patients using a black oocyte donor [76]. The same study reported lower pregnancy rates in black and Hispanic women undergoing IVF with donor oocyte compared with white women. There was no difference in clinical pregnancy rate between white and Asian recipients.

Embryo donation

Embryo donation has become more common as assisted reproduction has become more commonly utilized and more efficient, leading to the banking of many human embryos. Most often, donated embryos are obtained from couples who have successfully conceived through IVF and now wish to give their

cryopreserved supernumerary embryos to clinical programmes for use in infertile women [77]. If couples who have more embryos than they ultimately wish to use opt against donating their embryos, they are discarded. The ASRM most recently updated embryo donation guidelines in 2021. Recommendations include specifications regarding the cost of care, such that practices may charge patients for services such as thawing, embryo transfer, and cycle coordination, but there must be no fee paid for the embryo itself [78]. In this sense, the financial aspect of embryo donation is far stricter than that of oocyte donation. Additionally, gamete donors should undergo complete screening for infectious diseases and should provide their medical and genetic histories to recipients. If it is not possible to screen the donors or they do not consent to screening, the embryos remain eligible for use, but recipients must be thoroughly informed and counselled regarding the lack of testing [78]. Proper documentation of chain of custody of donated embryos and witnessed written relinquishment of embryos is also required. Finally, it is suggested that prospective embryo donors consider consultation with a psychological counsellor, as the decision to proceed with embryo donation can be a difficult one and it is critical that donors have fully considered the implications of doing so.

Future directions

The next frontier in oocyte donation includes the use of enucleated donor oocytes, which could permit recipients to use their own genetic material. At present time, mitochondrial replacement therapy (MRT) or “three-parent IVF” has already been successfully performed and could potentially allow for eradication of mitochondrial diseases [79]. By removing the nuclear DNA from an oocyte with abnormal mitochondrial DNA (mtDNA) into a fertilized donor oocyte, the embryo would have nuclear DNA from each parent and mtDNA from a donor. However, this technology is rife with controversy and has been banned in the United States over concerns regarding genetic modification of human gametes [80]. Additionally, it has been argued that the benefit gained from this intervention is not worth the risk to oocyte donors who undergo invasive medical procedures solely to donate oocytes for MRT, and it introduces a new element of uncertain legal implications [81]. As a last note, we should mention that there is no consensus among providers who have DIVF programmes as to what an evidence-based approach to management of oocyte donors and recipients is [82]. Given the controversies inherent to gamete donation, a closer examination of practices would be beneficial in the future to standardize our care.

References

- Hasler JF. Current status and potential of embryo transfer and reproductive technology in dairy cattle. *J Dairy Sci*. 1992;75(10):2857–79.
- Buster JE, Bustillo M, Thorneycroft IH, Simon JA, Boyers SP, Marshall JR, Louw JA, Seed RW, Seed RG. Non-surgical transfer of *in vivo* fertilised donated ova to five infertile women: Report of two pregnancies. *Lancet*. 1983;2(8343):223–4.
- Bustillo M, B J, Cohen SW, Hamilton F, Thorneycroft IH, Simon JA, Rodi IA, Boyers SP, Marshall JR, Louw JA. Delivery of a healthy infant following nonsurgical ovum transfer. *JAMA*. 1984;251(7):889.
- Trounson A, Leeton J, Besanko M, Wood C, Conti A. Pregnancy established in an infertile patient after transfer of a donated embryo fertilised *in vitro*. *Br Med J (Clin Res Ed)*. 1983;286(6368):835–8.
- Prevention CfDCa. 2019 Assisted Reproductive Technology Fertility Clinic Success Rates Report Atlanta: U.S. Department of Health and Human Services; 2019.
- Bustillo M, Buster JE, Cohen SW, Thorneycroft IH, Simon JA, Boyers SP, et al. Nonsurgical ovum transfer as a treatment in infertile women. Preliminary experience. *JAMA*. 1984;251(9):1171–3.
- Sullivan SD, Sarrel PM, Nelson LM. Hormone replacement therapy in young women with primary ovarian insufficiency and early menopause. *Fertil Steril*. 2016;106(7):1588–99.
- Ayesha, Jha V, Goswami D. Premature ovarian failure: An association with autoimmune diseases. *J Clin Diagn Res*. 2016 Oct; 10(10):QC10–QC12.
- Lindhardt Johansen M, Hagen CP, Rajpert-De Meyts E, Kjaergaard S, Petersen BL, Skakkebaek NE, et al. 45,X/46,XY mosaicism: Phenotypic characteristics, growth, and reproductive function—a retrospective longitudinal study. *J Clin Endocrinol Metab*. 2012;97(8):E1540–9.
- Chevalier N, Letur H, Lelannou D, Ohl J, Cornet D, Chalas-Boissonnas C, et al. Materno-fetal cardiovascular complications in turner syndrome after oocyte donation: Insufficient prepregnancy screening and pregnancy follow-up are associated with poor outcome. *J Clin Endocrinol Metab*. 2011;96(2):E260–7.
- Anasti JN. Premature ovarian failure: An update. *Fertil Steril*. 1998;70(1):1–15.
- Fortuno C, Labarta E. Genetics of primary ovarian insufficiency: A review. *J Assist Reprod Genet*. 2014;31(12):1573–85.
- Allen EG, Charen K, Hipp HS, Shubeck L, Amin A, He W, et al. Refining the risk for fragile X-associated primary ovarian insufficiency (FXPOI) by FMR1 CGG repeat size. *Genet Med*. 2021;23(9):1648–55.
- Sun B, Yeh J. Onco-fertility and personalized testing for potential for loss of ovarian reserve in patients undergoing chemotherapy: Proposed next steps for development of genetic testing to predict changes in ovarian reserve. *Fertil Res Pract*. 2021;7(1):13.
- Assisted Reproductive Technology National Summary Report. 2019. <https://archive.cdc.gov/#/details?url=https://www.cdc.gov/art/reports/2019/pdf/2019-Report-ART-Fertility-Clinic-National-Summary-h.pdf>
- Ethics Committee of the American Society for Reproductive Medicine. Oocyte or embryo donation to women of advanced reproductive age: An ethics committee opinion. *Fertil Steril*. 2016;106(5):e3–e7.
- Practice Committee of the American Society for Reproductive Medicine. Testing and interpreting measures of ovarian reserve: A committee opinion. *Fertil Steril*. 2020;114(6):1151–7.
- Martinez F, Racca A, Rodriguez I, Polyzos NP. Ovarian stimulation for oocyte donation: A systematic review and meta-analysis. *Hum Reprod Update*. 2021;27(4):673–96.
- Nelson SM, Klein BM, Arce JC. Comparison of antimullerian hormone levels and antral follicle count as predictor of ovarian response to controlled ovarian stimulation in good-prognosis patients at individual fertility clinics in two multicenter trials. *Fertil Steril*. 2015;103(4):923–30 e1.
- Iliodromiti S, Anderson RA, Nelson SM. Technical and performance characteristics of anti-Mullerian hormone and antral follicle count as biomarkers of ovarian response. *Hum Reprod Update*. 2015;21(6):698–710.
- Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology. Intracytoplasmic sperm injection (ICSI) for non-male factor indications: A committee opinion. *Fertil Steril*. 2020;114(2):239–45.
- Remohi J, Gallardo E, Levy M, Valbuena D, de los Santos MJ, Simon C, et al. Oocyte donation in women with recurrent pregnancy loss. *Hum Reprod*. 1996;11(9):2048–51.
- Ameratunga D, Weston G, Osianlis T, Catt J, Vollenhoven B. In vitro fertilisation (IVF) with donor eggs in post-menopausal women: Are there differences in pregnancy outcomes in women

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- with premature ovarian failure (POF) compared with women with physiological age-related menopause? *J Assist Reprod Genet.* 2009;26(9-10):511–4.
24. Krieg SA, Henne MB, Westphal LM. Obstetric outcomes in donor oocyte pregnancies compared with advanced maternal age in in vitro fertilization pregnancies. *Fertil Steril.* 2008;90(1):65–70.
 25. Storgaard M, Loft A, Bergh C, Wennerholm UB, Soderstrom-Anttila V, Romundstad LB, et al. Obstetric and neonatal complications in pregnancies conceived after oocyte donation: A systematic review and meta-analysis. *BJOG.* 2017;124(4):561–72.
 26. Munne S, Chen S, Colls P, Garrisi J, Zheng X, Cekleniak N, et al. Maternal age, morphology, development and chromosome abnormalities in over 6000 cleavage-stage embryos. *Reprod Biomed Online.* 2007;14(5):628–34.
 27. Bracewell-Milnes T, Saso S, Bora S, Ismail AM, Al-Memar M, Hamed AH, et al. Investigating psychosocial attitudes, motivations and experiences of oocyte donors, recipients and egg sharers: A systematic review. *Hum Reprod Update.* 2016;22(4):450–65.
 28. Ethics Committee of the American Society for Reproductive Medicine. Access to fertility treatment irrespective of marital status, sexual orientation, or gender identity: An ethics committee opinion. *Fertil Steril.* 2021;116(2):326–30.
 29. Ethics Committee of the American Society for Reproductive Medicine. Access to fertility services by transgender and non-binary persons: An ethics committee opinion. *Fertil Steril.* 2021;115(4):874–8.
 30. Pasch LA, Sullivan KT. Stress and coping in couples facing infertility. *Curr Opin Psychol.* 2017;13:131–5.
 31. Kirkman-Brown JC, Martins MV. ‘Genes versus children’: If the goal is parenthood, are we using the optimal approach? *Hum Reprod.* 2020;35(1):5–11.
 32. Hauzman EE, Garcia-Velasco JA, Pellicer A. Oocyte donation and endometriosis: What are the lessons? *Semin Reprod Med.* 2013;31(2):173–7.
 33. Ng KYB, Cheong Y. Hydrosalpinx - salpingostomy, salpingectomy or tubal occlusion. *Best Pract Res Clin Obstet Gynaecol.* 2019;59:41–7.
 34. Kasius A, Smit JG, Torrance HL, Eijkemans MJ, Mol BW, Opmeer BC, et al. Endometrial thickness and pregnancy rates after IVF: A systematic review and meta-analysis. *Hum Reprod Update.* 2014;20(4):530–41.
 35. Mouhayar Y, Franasiak JM, Sharara FI. Obstetrical complications of thin endometrium in assisted reproductive technologies: A systematic review. *J Assist Reprod Genet.* 2019;36(4):607–11.
 36. Glujsovsky D, Pesce R, Suelo C, Quinteiro Retamar AM, Hart RJ, Ciapponi A. Endometrial preparation for women undergoing embryo transfer with frozen embryos or embryos derived from donor oocytes. *Cochrane Database Syst Rev.* 2020;10: CD006359.
 37. Dyer C. Payment to egg donors is to be tripled to remedy shortage. *BMJ.* 2011;343:d6865.
 38. Kool EM, Bos AME, van der Graaf R, Fauser B, Bredenoord AL. Ethics of oocyte banking for third-party assisted reproduction: A systematic review. *Hum Reprod Update.* 2018;24(5):615–35.
 39. Hinkson L, Dame C, Braun T, Nachtigall I, Henrich W. Never too late? Quadruplets at the age of 65 years. *Arch Gynecol Obstet.* 2021;304(4):851–4.
 40. Ethics Committee of the American Society for Reproductive Medicine. Financial compensation of oocyte donors: An ethics committee opinion. *Fertil Steril.* 2021;116(2):319–25.
 41. Frith L. Gamete donation, identity, and the offspring’s right to know. *Virtual Mentor.* 2007;9(9):644–8.
 42. Sauer MV. Further HFEA restrictions on egg donation in the UK: Two strikes and you’re out! *Reprod Biomed Online.* 2005;10(4): 431–3.
 43. McKenzie L, Sangi-Haghpeykar H, Gibbons W. Are commercial egg banks all they are cracked up to be? *Fertil Steril.* 2022;117(2):349–50.
 44. Yeh JS, Steward RG, Dude AM, Shah AA, Goldfarb JM, Muasher SJ. Pregnancy rates in donor oocyte cycles compared to similar autologous in vitro fertilization cycles: An Analysis of 26,457 fresh cycles from the society for assisted reproductive technology. *Fertil Steril.* 2014;102(2):399–404.
 45. Kushnir VA, Darmon SK, Barad DH, Gleicher N. New national outcome data on fresh versus cryopreserved donor oocytes. *J Ovarian Res.* 2018;11(1):2.
 46. Williams RS, Ellis DD, Wilkinson EA, Kramer JM, Datta S, Guzik DS. Factors affecting live birth rates in donor oocytes from commercial egg banks vs. program egg donors: An Analysis of 40,485 cycles from the society for assisted reproductive technology registry in 2016-2018. *Fertil Steril.* 2022;117(2):339–48.
 47. Insogna IG, Lanes A, Lee MS, Ginsburg ES, Fox JH. Association of fresh embryo transfers compared with cryopreserved-thawed embryo transfers with live birth rate among women undergoing assisted reproduction using freshly retrieved donor oocytes. *JAMA.* 2021;325(2):156–63.
 48. Cobo A, Meseguer M, Remohi J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: A prospective, randomized, controlled, clinical trial. *Hum Reprod.* 2010;25(9):2239–46.
 49. Maxwell KN, Cholst IN, Rosenwaks Z. The incidence of both serious and minor complications in young women undergoing oocyte donation. *Fertil Steril.* 2008;90(6):2165–71.
 50. Humaidan P, Kol S, Papanikolaou EG; Copenhagen GnRH ATWG. GnRH agonist for triggering of final oocyte maturation: Time for a change of practice? *Hum Reprod Update.* 2011;17(4):510–24.
 51. Hoyos LR, Cheng CY, Brennan K, Hubert G, Wang B, Buyalos RP, et al. Euploid rates among oocyte donors: Is there an optimal age for donation? *J Assist Reprod Genet.* 2020;37(3):589–94.
 52. Hogan RG, Wang AY, Li Z, Hammarberg K, Johnson L, Mol BW, et al. Oocyte donor age has a significant impact on oocyte recipients’ cumulative live-birth rate: A population-based cohort study. *Fertil Steril.* 2019;112(4):724–30.
 53. Martinez F, Kava-Braverman A, Clua E, Rodriguez I, Gaggiotti Marre S, Coroleu B, et al. Reproductive outcomes in recipients are not associated with oocyte donor body mass index up to 28 kg/m²: A cohort study of 2722 cycles. *Reprod Biomed Online.* 2017;35(6):739–46.
 54. Freour T, Massart P, Garcia D, Vassena R, Rodriguez A. Revisiting the association between smoking and female fertility using the oocyte donation model. *Reprod Biomed Online.* 2018;37(5):564–72.
 55. Brown S. Genetic aspects of donor selection. In: Sauer M, editor. *Principles of Oocyte and Embryo Donation.* New York, NY: Springer-Verlag; 1998. pp. 53–63.
 56. Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology. Recommendations for gamete and embryo donation: A committee opinion. *Fertil Steril.* 2013;99(1): 47–62.e1.
 57. Committee Opinion No. 690. Carrier screening in the age of genomic medicine. *Obstet Gynecol.* 2017;129(3):e35–e40.
 58. Burke CA, Howell D, Cortes J, Skytte A, Fernandez R, Pixley S. (eds.). An improved method for Zika virus screening in a gamete bank setting. In: 76th American Society for Reproductive Medicine Scientific Congress & Expo. Virtual: Fertility and Sterility; 2020.
 59. Mackens S, Santos-Ribeiro S, van de Vijver A, Racca A, Van Landuyt L, Tournaye H, et al. Frozen embryo transfer: A review on the optimal endometrial preparation and timing. *Hum Reprod.* 2017;32(11):2234–42.
 60. Soares SR, Troncoso C, Bosch E, Serra V, Simon C, Remohi J, et al. Age and uterine receptiveness: Predicting the outcome of oocyte donation cycles. *J Clin Endocrinol Metab.* 2005;90(7): 4399–404.
 61. Madero S, Rodriguez A, Vassena R, Verneve V. Endometrial preparation: Effect of estrogen dose and administration route on reproductive outcomes in oocyte donation cycles with fresh embryo transfer. *Hum Reprod.* 2016;31(8):1755–64.

62. Twigt JM, Hammiche F, Sinclair KD, Beckers NG, Visser JA, Lindemans J, et al. Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation. *J Clin Endocrinol Metab.* 2011;196(2):E322–9.
63. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G, et al. Effect of b vitamins and genetics on success of in-vitro fertilisation: Prospective cohort study. *Lancet.* 2006;367(9521):1513–9.
64. Liu Y, Wu Y. Progesterone intramuscularly or vaginally administration May not change live birth rate or neonatal outcomes in artificial frozen-thawed embryo transfer cycles. *Front Endocrinol (Lausanne).* 2020;11:539427.
65. Devroey P, Camus M, Palermo G, Smitz J, Van Waesberghe L, Wisanto A, et al. Placental production of estradiol and progesterone after oocyte donation in patients with primary ovarian failure. *Am J Obstet Gynecol.* 1990;162(1):66–70.
66. Pan SP, Chao KH, Huang CC, Wu MY, Chen MJ, Chang CH, et al. Early stop of progesterone supplementation after confirmation of pregnancy in IVF/ICSI fresh embryo transfer cycles of poor responders does not affect pregnancy outcome. *PLoS One.* 2018;13(8):e0201824.
67. Practice Committee of the American Society for Reproductive Medicine and the Practice Committee for the Society for Assisted Reproductive Technologies. Guidance on the limits to the number of embryos to transfer: A committee opinion. *Fertil Steril.* 2021;116(3):651–4.
68. Fransasiak JM, Forman EJ, Hong KH, Werner MD, Upham KM, Treff NR, et al. The nature of aneuploidy with increasing age of the female partner: A review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertil Steril.* 2014;101(3):656–63 e1.
69. Doyle N, Gainty M, Eubanks A, Doyle J, Hayes H, Tucker M, et al. Donor oocyte recipients do not benefit from preimplantation genetic testing for aneuploidy to improve pregnancy outcomes. *Hum Reprod.* 2020;35(11):2548–55.
70. Ubaldi FM, Cimadomo D, Vaiarelli A, Fabozzi G, Venturella R, Maggiulli R, et al. Advanced maternal age in IVF: Still a challenge? The present and the future of its treatment. *Front Endocrinol (Lausanne).* 2019;10:94.
71. Yeh JS, Steward RG, Dude AM, Shah AA, Goldfarb JM, Muasher SJ. Pregnancy outcomes decline in recipients over age 44: An Analysis of 27,959 fresh donor oocyte in vitro fertilization cycles from the society for assisted reproductive technology. *Fertil Steril.* 2014;101(5):1331–6.
72. Rizzello F, Coccia ME, Fatini C, Badolato L, Fantappie G, Merrino V, et al. Comorbidities, risk factors and maternal/perinatal outcomes in oocyte donation pregnancies. *Reprod Biomed Online.* 2020;41(2):309–15.
73. Jeve YB, Potdar N, Opoku A, Khare M. Donor oocyte conception and pregnancy complications: A systematic review and meta-analysis. *BJOG.* 2016;123(9):1471–80.
74. McCarter K, Setton R, Chung A, An A, Rosenwaks Z, Spandorfer S. Is increasing paternal age negatively associated with donor oocyte recipient success? A paired analysis using sibling oocytes. *Fertil Steril.* 2021;116(2):373–9.
75. Dviri M, Madjunkova S, Koziarz A, Antes R, Abramov R, Mashiah J, et al. Is there a correlation between paternal age and aneuploidy rate? An analysis of 3,118 embryos derived from young egg donors. *Fertil Steril.* 2020;114(2):293–300.
76. Zhou X, McQueen DB, Schufreider A, Lee SM, Uhler ML, Feinberg EC. Black recipients of oocyte donation experience lower live birth rates compared with white recipients. *Reprod Biomed Online.* 2020;40(5):668–73.
77. Hallich O. Embryo donation or embryo adoption? Conceptual and normative issues. *Bioethics.* 2019;33(6):653–60.
78. Practice Committee of the American Society for Reproductive Medicine and the Practice Committee for the Society for Assisted Reproductive Technology. Guidance regarding gamete and embryo donation. *Fertil Steril.* 2021;115(6):1395–410.
79. Palacios-Gonzalez C. Mexico and mitochondrial replacement techniques: What a mess. *Br Med Bull.* 2018;128(1):97–107.
80. Pompei M, Pompei F. Overcoming bioethical, legal, and hereditary barriers to mitochondrial replacement therapy in the USA. *J Assist Reprod Genet.* 2019;36(3):383–93.
81. Palacios-Gonzalez C. Mitochondrial replacement techniques: Egg donation, genealogy and eugenics. *Monash Bioeth Rev.* 2016;34(1):37–51.
82. Weissman A, Leong M, Sauer MV, Shoham Z. Characterizing the practice of oocyte donation: A web-based international survey. *Reprod Biomed Online.* 2014;28(4):443–50.

GESTATIONAL SURROGACY

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History

Gestational surrogacy is considered one of the entities of third-party reproduction, which also includes gamete or embryo donation and adoption. We have witnessed a significant change in media exposure and cultural attitude to the topic, as key figures in social media candidly shared their experience with surrogacy. Thus, once taboo to some, this option has gradually been employed more often, and as such our need to familiarize with all aspects of surrogacy arises.

Surrogacy is not new to the 21st century. In fact, third-party reproduction dates even to the book of Genesis in the Old Testament of the bible, in which “natural surrogacy” is described as practiced by Abraham, Sarah, and their servant Hagar. This is probably one of many tales of women bearing children for kings and rulers whose spouses could not carry pregnancy. With the development of artificial insemination techniques, this became a more socially acceptable means rather than “natural” means employed. From a legal perspective, a formal legal surrogacy agreement was first drafted in 1976 by Noel Keane, a US lawyer, and strong advocate for surrogacy, involved in the birth of approximately 600 children via surrogacy throughout his career. Yet, the first legally compensated surrogate was Elizabeth Kane, in 1980, marking the first case of formal monetarily compensation. Natural surrogacy carried with it difficulties, and as such was a source for legal dispute in several well-recognized legal battles, including the case of “Baby M” [1] and Smith versus Jones [2]. 1978 marked the birth of the first baby by *in vitro* fertilization (IVF), Louise Brown, and with it the notion that embryos created entirely from the gametes of the “genetic couple” could be transferred to the “gestational carrier,” who therefore provided no genetic contribution to any child that resulted from the arrangement. The first gestational surrogacy in the United Kingdom and United States took place in 1985, in which Kim Cotton and Shannon Boff, respectively, carried a child genetically unrelated to them on behalf of the intended parents.

Gestational surrogacy is now accepted in the United Kingdom as a treatment option for infertile couples, provided there are clearly defined medical indications. A report commissioned by the British Medical Association (BMA) in 1990 [3] provided the first evidence that surrogacy was formally accepted as a legitimate treatment option. In the same year, the Human Fertilization and Embryology Act (1990) [4] was passed through the UK Parliament and did not ban surrogacy. The most recent report of the BMA [5] states that “surrogacy is an acceptable option of last resort in cases where it is impossible or highly undesirable for medical reasons for the intended mother to carry a child herself.”

Previously, surrogacy was practiced in only a limited number of IVF centres in the United Kingdom, so that in a 1998 report from Bourn Hall, UK, treatment by surrogacy accounted for 1% of the total annual throughput of cases out of a total of about 2500 IVF and frozen embryo replacement cycles [6]. Current estimations

are more optimistic, and a 2015 report described that among participating clinics in the survey, 42.6% offered surrogacy [7], with heterosexual couples as the largest group using services, followed by male same-sex couples. Yet, accessibility to surrogacy is not similar globally. During the years of this protracted debate in the United Kingdom, most other European countries had decided to ban the practice of surrogacy of any kind. In a 2022 worldwide survey on behalf of the International Federation of Fertility Societies (IFFS), it was reported that of the 29 countries surveyed, only 28% allowed and/or practiced surrogacy [8]. The largest experience to date of both natural and gestational surrogacy is in the United States, where commercial surrogacy arrangements are allowed and 90% of clinics offer gestational surrogacy [9]. In 2019, 1.9% of assisted reproductive technology (ART) cycles included gestational carriers, and the number and percentage of embryo transfers with a gestational carrier increased from 2649 (2.1%) to 9195 (5.4%) from 2010 to 2019, respectively [9].

The history of surrogacy raises with it ethical considerations, and clearly past norms and practices are non-relevant or ethical to modern times. Modern surrogacy involves voluntary participation of a woman willing to serve as a gestational carrier for couples who cannot carry a pregnancy. As physicians, it mandates our immaculate supervision to ensure safety and wellness of all sides involved. This chapter aims to review all relevant medical and non-medical aspects related to gestational surrogacy.

Definitions and terms

Surrogacy involves a woman carrying a gestation for an intended couple, and as such she is often termed the gestational carrier. In “gestational surrogacy,” “full surrogacy,” or “IVF surrogacy,” the gametes of the “genetic couple,” “commissioning couple,” or “intended parents” in a surrogacy arrangement are used to produce embryos, and these embryos are subsequently transferred to a woman who agrees to act as a carrier for these embryos. The “gestational carrier” is therefore genetically unrelated to any offspring that may be born as a result of this arrangement. With “natural surrogacy” or “partial surrogacy,” the gestational carrier is inseminated with the semen of the husband of the “genetic couple.” Any resulting child is therefore genetically related to the carrier. In this chapter, only treatment by “gestational surrogacy” will be discussed, and the couple who initiates the surrogacy arrangement and whose gametes are used will be known as the “genetic couple” and the woman who subsequently carries the child will be known as the “gestational carrier.”

Indications

Genetic couples in need of a gestational carrier can be divided to two groups—those without a uterus and those with a uterus, as detailed in this chapter. In those without a uterus, cases may be further divided to congenital or acquired state. Congenital

absence of the uterus is known as Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome, while the acquired form follows hysterectomy due to various pathologies. Same-sex male couples would also be associated with this group (lack of uterus). Patients with a uterus but in need of a gestational carrier include cases following repeated implantation failure in IVF, repeated pregnancy loss, and various underlying maternal conditions and past severe obstetric complications, in which pregnancy is medically contraindicated. These may include renal failure, antiphospholipid antibody syndrome with systemic involvement, complicated systemic lupus erythematosus, and severe heart disease, such as maternal cardiomyopathy. Other non-frequent underlying diseases are severe recurrent pre-eclamptic toxæmia, past placenta accreta, and post-transplantation status, although it is beyond the scope of this chapter to note every potential indication.

In a cohort from Mexico, where only altruistic surrogacy is allowed, 32% of cases were following hysterectomy, 21% following repeat implantation failure, 21% for couples with no female partner, 19% were for maternal medical conditions or previous obstetric complications, 11% for repeat pregnancy loss, and 3% for uterine pathologies [10]. In a separate Canadian cohort, after the exclusion of same-sex male couples and single males, 47.0% were described as unable to carry a pregnancy, due to repeated implantation failure, repeated pregnancy loss, and previous poor pregnancy outcomes, while 53.0% were described as unable to carry a pregnancy due to severe Asherman's syndrome, uterine malformations or agenesis, and maternal medical comorbidities [11]. Finally, in a case series from Israel, lack of uterus was the indication for surrogacy in 52.6% of cases, including MRKH syndrome and patients post hysterectomy, while 47.4% of cases were due to repeated implantation failure, repeat pregnancy loss, and maternal medical condition [12].

Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome

As Mayer–Rokitansky–Küster–Hauser syndrome (MRKHS) is a relatively frequent indication for gestational surrogacy, it is discussed in further detail as follows. MRKHS is a congenital anomaly of the genital tract with an incidence of 1:4900 female births [13]. Most commonly, an investigation for primary amenorrhea will lead to diagnosis in adolescence. The syndrome consists of complete absence of the uterus or a rudimentary uterus consisting of two small bilateral fibromuscular remnants, with vaginal aplasia. Fallopian tubes are present as are functioning ovaries, as the source for embryonal development differs [14]. The karyotype as a rule is 46XX, and the secondary sex characteristics are usually feminine. MRKHS is frequently associated with urinary, skeletal, and cardiac defects. Urinary tract anomalies include renal agenesis/aplasia, pelvic kidney, horseshoe kidney, renal sclerosis, and double ureter [15]. The genetic origins of MRKHS are diverse and include copy number variations and point mutations [16], with recent research suggesting a more common involvement of a limited number of genes among tens of candidate genes previously identified [17].

Duncan et al. proposed the term MURCS (Malformations Urinary Cardiac and Skeletal) for cases where systemic involvement was present [18]. This description was later incorporated in more updated classification by Oppelt et al. [19]. As based on a series of 53 MRKHS patients, the authors created a clinical diagnostic classification of the syndrome: the typical form in which the fallopian tubes, ovaries, and renal system are generated and well developed; and the atypical form in which additional malformations of the ovaries and/or the renal system are present [19].

Past medical focus on these patients addressed issues of quality of life, such as vaginal reconstruction to enable sexual intercourse. Yet, with advances in reproductive medicine, the possibility for motherhood via a gestational carrier became possible [20], and, more lately, via potential uterine transplant [21], as discussed in Chapter 66.

The IVF performance of MRKHS patients has been found different in the two subtypes mentioned previously. Based on follow-up data on a total of 102 cycles of surrogate IVF in 27 MRKHS patients, women with the typical form of MRKHS require less gonadotropins and a shorter duration of ovarian stimulation. The mean number of follicles, oocytes, and metaphase II oocytes, the fertilization rate, and cleaving embryos were higher among women with the typical form. Yet eventually, pregnancy rates were similar since the available number and quality of transferred embryos to the surrogate mothers was not affected [22].

Limited data to date to assess the potential genetic transmission of congenital abnormalities to female offspring seems reassuring, as in a report of 34 live-born children to patients with MRKHS, half of whom were female, no congenital anomalies were found [23].

Patient selection

Assessment and counselling of intended parents

The intended couple will usually have undergone assessment in context of previous failed IVF treatments, but less so in cases in which treatment is to be initiated for the first time with a gestational carrier. Assessment will thus consist primarily of an evaluation for the need for a gestational carrier, in accordance with indications already listed, or others deems relevant.

Suitability for hormonal treatment and subsequent collection as part of IVF should be applied. In patients in which surrogacy is indicated due to MRKHS, this may include assessment for renal anomalies to identify a displaced kidney (for oocyte collection consideration), while in patients who underwent previous hysterectomy due to a premalignant state, evaluation should best include clearance from their attending oncologist prior to hormonal administration. For patients in need of a gestational carrier due to maternal comorbidities, baseline health and eligibility for IVF are best assessed on a personal basis, as for any medications patients may be taking regularly. For patients with repeated implantation failure/pregnancy loss, an evaluation on parental karyotype is best confirmed, if not already performed, to avoid similar results with a surrogate.

As for any couple undergoing IVF, evaluation of ovarian reserve markers is advised for protocol adjustment, with preferable ultrasound to assess pelvic anatomy. Baseline testing should be employed as based on local regulations, and will usually consist of a physical examination, a recent pap test, testing for hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) status, and a complete blood count [24].

For the male, a semen analysis should be employed to rule out the rare cases of previously unsuspected azoospermia, and to assess the need for intracytoplasmic sperm injection. The blood groups of the genetic parents are requested in case the gestational carrier is rhesus negative.

Legal aspects should be considered, as dependent on local restrictions. For example, in Israel, gestational surrogacy may be employed only if oocytes were obtained from the intended mother when she was younger than 41. Legal and ethical considerations must also be taken into account when considering the

need for surrogacy in intended couples with malignancies, as to ensure sufficient parental life expectancy.

Finally, in depth counselling must be provided to the intended couple, either by the centre's psychologist or other designated personnel. Points to address include, but are limited to, the following [25]:

- A review of all alternative treatment options [26].
- The need to find their own gestational carrier (UK), practical cost of treatment by gestational surrogacy, and need for insurance coverage for the gestational carrier.
- The importance of obtaining legal advice, and the need to establish a contract with the gestational carrier which addresses all legal aspects of the gestational surrogacy. This includes reference to the possibility that the gestational carrier may wish to retain the child after birth, as legally addressed by different countries. In addition, an agreement should lay the base for future interactions between the intended parents and gestational carrier, with reference to future degree of involvement that the gestational carrier may wish to have with the child.
- Psychological aspects posed by surrogacy, including potential psychological effect to the child. The intended parents are best informed of their option to receive psychological support for this process, starting from the decision to seek a gestational carrier.
- Medical risks associated with the process, including the risk for multiple gestation, the possibility that a child may be born with a handicap, and all IVF-associated risks.

Assessment of the gestational carrier

Regulations for gestational carriers vary worldwide. This includes minimal age requirement, the ability to act as gestational carrier for family members, medical considerations, and financial reimbursement. Even when allowed under law, the situation in which family members offer to serve as gestational carriers should be carefully assessed for unhealthy family dynamics and undue pressure being employed. Thus, an in-length interview with the potential gestational carrier is advised. The Ethics Committee in the United Kingdom has recommended that gestational carriers should be married and that the husband or partner should be made fully aware during the counselling process of the implications of his partner acting as a gestational carrier.

Medical considerations vary but must consider the health of the potential gestational carrier, prior delivery modes and obstetric complications and psychological health [27]. A baseline evaluation should preferably include a physical examination, sonographic pelvic evaluation with emphasis to the uterus, and testing for common infections for both the intended gestational carrier and her partner, including HBV, HCV, and HIV.

Points to address include, but are not limited to, the following [28]:

- The possibility of family and friends being against such treatment
- The normal medical risks associated with pregnancy including an abortion, ectopic pregnancy, premature delivery, and the possibility of caesarean section
- The full implications of undergoing treatment by IVF surrogacy
- The possibility of multiple pregnancy including fetal reduction

- The possibility that the gestational carrier will feel a sense of bereavement when she gives the baby to the genetic couple

Choice of treatment protocol

The choice of protocol employed must take into account availability of the gestational carrier and intended parents, eligibility of the gestational carrier for protocol selection (menses regularity for example), and local regulations. In the United Kingdom, the embryos obtained from the genetic couple must be frozen for a six-month "quarantine" period pending a repeat negative HIV report prior to their transfer to the gestational carrier [29]. Thus, consideration is mainly aimed at choice of frozen embryo transfer protocol for the gestational carrier.

If synchronization of the intended parents and gestational carrier is considered to enable a fresh transfer, the gestational carrier may be prescribed oral contraceptive pills and short-term gonadotropin-releasing hormone (GnRH) agonists as needed, until stimulation is started for the genetic mother. If programmed transfer is planned, a mock cycle may precede, to ensure average response time to oestrogen priming. Natural cycle transfer may also be considered, although probably less flexible in regard to fresh transfer, and entails the possibility of a spontaneous pregnancy by the gestational carrier. Stimulation of the genetic mother is personalized, although preference to GnRH antagonist protocols with GnRH agonist triggering should be given (see Chapter 46 on ovarian stimulation for freeze-all cycles) [30]. The intended parents may opt for pregestational testing for aneuploidy to enhance probability for a transfer of a genetically healthy embryo, although this has not been demonstrated to increase live births [31].

If no synchronization of the patients is required, the choice of programmed versus natural thawed-frozen embryo transfer should be considered and debated with the patient. Many, however, consider the use of a natural ovulatory cycle for timing FET unacceptable because of the risk for a spontaneous pregnancy by the gestational carrier.

Finally, the choice of single versus multiple embryo transfer must be discussed. Transfer of more than one embryo is associated with an increase in clinical pregnancies, live births, and multiple gestations. Yet, multiple gestations entail a higher miscarriage rate and higher rate of adverse obstetric outcomes, such as preterm births and low birthweight [31, 32].

Results

Treatment by gestational surrogacy generally achieves satisfactory pregnancy and live birth rates per genetic couple and per gestational carrier. To date, a limited number of series have been reported in the literature. In the original series reported by Utian and colleagues [33], a clinical pregnancy rate of 18% (7/59) per cycle initiated and a 23% clinical pregnancy rate per ET was achieved. A later series of 180 cycles of IVF gestational surrogacy, reported by the same group, gave an overall pregnancy rate per cycle of 24% and a live birth rate of 15.8% [34]. Corson and colleagues reported a clinical pregnancy rate of 58% per intended couple and 33.2% per ET in women where the genetic mothers were less than 40 years of age [35], while another larger series from the United States showed ongoing or delivered pregnancy rates of 36% [36]. In Born Hall, UK, live birth rates of between 37% and 43% per genetic couple and 34% and 39% per gestational

carrier have been achieved, with a mean of two embryos transferred [29, 37]. In a recent report of 170 embryo transfers to 81 gestational carriers, the vast majority of which were single thawed-frozen transfers, live birth rate was 23.5% for the first transfer, and the cumulative rate was 50.6% after the sixth cycle [38]. In an additional report from Australia, of 360 embryo transfer cycles, the rates of clinical pregnancy and live delivery were 26% and 19%, respectively, noting no difference between single and double embryo transfer [39].

Obstetric outcomes described depend most on the number of embryos transferred and resultant multiple pregnancies. As previously stated, the transfer of more than one embryo may increase probability for pregnancy and live birth, yet is associated with a higher rate of multiple pregnancies. These are high-risk pregnancies, associated with a significantly lower gestational age at delivery and higher rates of preterm births, and lower average birthweight and more low birthweight neonates [31, 39]. Gestational maternal morbidity seems comparable between gestational carriers and matched controls, and lower than that for the IVF population [40]. This includes hypertensive disorder rate and caesarean delivery rate. Yet, rates of placenta previa may be higher in gestational carriers than controls [41], as IVF has been shown to increase risk, independently of subfertility status [42].

Long-term psychological assessment of all sides involved in surrogacy is of interest. In a 2015 meta-analysis of surrogate pregnancies, the authors concluded that there were no major psychological differences between children conceived by surrogacy and other types of ART, as assessed at the age of 10, as there were no significant differences in psychological well-being between intended mothers, mothers following ART and following unassisted conceptions [41]. The authors also noted no differences in psychological parameters assessed between children of gestational carriers and controls, and in gestational carriers themselves, although some cases of difficulty in separation from the new-born were noted.

Additional considerations

The major ethical and practical problems that might be encountered with IVF surrogacy are described in the following subsections.

Religious attitudes

Religious attitudes towards surrogacy differ widely.

The Catholic Church is strongly against all forms of assisted conception, particularly those that involve gamete donation and surrogacy [43]. Therefore, surrogacy is banned in the Catholic countries of Europe: Italy, France, and others. The Anglican Church is less rigid in its view on surrogacy and has not condemned it.

The Jewish religion, which is very much family oriented and puts a duty on Jewish couples to have children, does not forbid the practice of gestational surrogacy [44]. From the religious point of view, a child born through gestational surrogacy to a Jewish couple will belong to the father who gave the sperm (therefore sperm donation is not allowed in gestational surrogacy in Israel) and to the woman who gave birth [45].

The Islamic view appears absolute and, in the same way that the use of donor gametes is strictly forbidden, surrogacy is not allowed. It is suggested that it may be permissible between wives in the same marriage, but the debate continues [46].

Compensation

The question of whether it is ethical to pay gestational carriers and, if so, how much, has always caused concern. In the United States and Israel, payment is “up front” and revealed, whereas in the UK and most of Europe, Australia, and New Zealand, altruistic surrogacy is what everyone aspires to, but it is in effect impractical, and payment is often labelled as “reasonable expenses.” Many also consider it unethical not to pay gestational carriers for the sacrifices that they make to help other couples. The European Society of Human Reproduction and Embryology (ESHRE) Task Force on Ethics and the Law (2005) [29] states that payment for (surrogacy) services is unacceptable, whereas the IFFS Surveillance Report 2010 [47] states only that “the payment to the surrogate raises special concerns.”

In treatment and neonatal care aspects

- Poor ovarian response of “genetic mothers” to stimulation may be encountered. In the post-hysterectomy cases, this reduced follicular response may be due to reduced vascular supply to the ovaries [48].
- The gestational carrier may wish to keep the child.
- An abnormal child may be rejected by both the genetic and gestational carrier parents.

Long-term post-treatment aspects

- The long-term effects on the children born as a result of gestational surrogacy are not known.
- The long-term psychological effects on both the “genetic couple” and “gestational carrier” are not known.
- The impact on the gestational carriers’ existing children, namely, the mother–child relationship has not been studied extensively. Golombok et al. found no difference in maternal negativity, maternal positivity, mother–child interaction, and child adjustment between surrogacy and egg donation compared with natural conception [49]. However, surrogacy and egg donation families showed less-positive mother–child interaction compared with natural conception.

Cross-border surrogacy

Because there are a number of leading countries, particularly in Europe, such as Italy, Germany, and France, where surrogacy is not permitted, and as the ease of worldwide travel increases, couples now travel for treatment that is unavailable in their own countries. Cross-border reproductive care, which means crossing borders to have children, is a rapidly growing phenomenon [50]. This has led to different issues which require our focus in the treatment of all sides involved, and especially in the face of the recent global pandemic [51].

Conclusion

The practice of gestational surrogacy can only be carried out in clinics licensed by the Human Embryology and Fertilization Act (HEFA) in the UK, and in selected countries in Europe, Israel, and in the United States.

The indications for treatment by gestational surrogacy are limited to a small group of women who have no uterus (congenital or acquired), suffer recurrent miscarriages or repeated implantation failure, or suffer from certain medical conditions that would threaten the life of a woman if she were to become pregnant, in

addition to same-sex male couples who wish to become parents. While considering the efficacy of gestational surrogacy, it is safe to assume that the treatment of young women with very specific indications is successful and relatively free of complications.

At the base of healthy surrogacy remains the extreme care with which the gestational carrier must be selected to ensure complete compatibility, and also the in-depth counselling that is required, both in the short and the long term, on all aspects of the treatment. The support and advice of an independent counsellor and lawyer are absolutely essential. As clinicians, we bear the responsibility of medical supervision to all sides involved, but should always ensure careful consideration of social, religious, or ethical aspects of treatment with surrogacy. We advise clinicians who are involved in treatments assisted by gestational surrogacy to refer to the most updated recommendations of practices, which provide a comprehensive review of screening, evaluation, psycho-educational and legal recommendations [52].

During our long-term experience both in Israel and in the UK, no serious clinical, ethical, or legal problems have been encountered. Yet, often, because the gestational carrier is healthy, young, and known to be fertile, she and the genetic parents invariably expect success, and may get discouraged if this is not achieved. Full support counselling for both couples is essential when this occurs. Gestational carrier services should be part of a comprehensive infertility treatment programme that larger centres offer, now that it is an ethically accepted form of treatment in numerous countries worldwide.

References

- Andrews LB. The Stork market: The law of the new reproduction technologies. *ABA J.* 1984;70(8):50–6.
- Annas GJ. Using genes to define motherhood—the California solution. *N Engl J Med.* 1992;326(6):417–20.
- British Medical Association. Surrogacy: Ethical Considerations. Report of the Working Party on Human Infertility Services. London, UK: BMA publications, 1990.
- Human Fertilisation and Embryology Act. London, UK: Her Majesty's Stationery Office, 1990.
- Association BM. Changing Conceptions of Motherhood. The Practice of Surrogacy in Britain. London, UK: BMA publications, 1996.
- Balen AH, Hayden CA. British fertility society survey of all licensed clinics that perform surrogacy in the UK. *Hum Fertil (Camb).* 1998;1(1):6–9.
- Norton W, Crawshaw M, Hudson N, Culley L, Law C. A survey of UK fertility clinics' approach to surrogacy arrangements. *Reprod Biomed Online.* 2015;31(3):327–38.
- Ory SJ. International Federation of Fertility Societies' Surveillance (IFFS) 2022: Global Trends in Reproductive Policy and Practice, 9th Edition [Internet]. 2022. Available from: <https://www.iffs-production.org/wp-content/uploads/2022/04/2022-Surveillance-Report-April-2-2022-Upload.pdf>
- Centers for Disease Control and Prevention. 2019 Assisted Reproductive Technology Fertility Clinic and National Summary Report. 2021.
- Cabra R, Alduncin A, Cabra JR, Ek LH, Briceño M, Mendoza PB. Gestational surrogacy. Medical, psychological and legal aspects: 9 years of experience in Mexico. *Hum Reprod Open.* 2018;2018(1):hox029.
- Dar S, Lazer T, Swanson S, Silverman J, Wasser C, Moskowitz SI, et al. Assisted reproduction involving gestational surrogacy: An Analysis of the medical, psychosocial And legal issues: Experience from a large surrogacy program. *Hum Reprod.* 2015 Feb;30(2):345–52.
- Raziel A, Schachter M, Strassburger D, Komarovsky D, Ron-El R, Friedler S. Eight years' experience with an IVF surrogate gestational pregnancy programme. *Reprod Biomed Online.* 2005;11(2):254–8.
- Herlin M, Bjørn A-MB, Rasmussen M, Trolle B, Petersen MB. Prevalence and patient characteristics of Mayer-Rokitansky-Küster-Hauser syndrome: A nationwide registry-based study. *Hum Reprod.* 2016;31(10):2384–90.
- Choussein S, Nasioudis D, Schizas D, Economopoulos KP. Müllerian dysgenesis: A critical review of the literature. *Arch Gynecol Obstet.* 2017;295(6):1369–81.
- Chen N, Pan H, Luo G, Wang P, Xie Z, Hua K, et al. Clinical characteristics of 1,055 Chinese patients with Mayer-Rokitansky-Küster-Hauser syndrome: A nationwide multicentric study. *Fertil Steril.* 2021 Aug;116(2):558–65.
- Williams LS, Demir Eksi D, Shen Y, Lossie AC, Chorich LP, Sullivan ME, et al. Genetic analysis of Mayer-Rokitansky-Kuster-Hauser syndrome in a large cohort of families. *Fertil Steril.* 2017 Jul;108(1):145–151.e2.
- Mikhael S, Dugar S, Morton M, Chorich LP, Tam KB, Lossie AC, et al. Genetics of agenesis/hypoplasia of the uterus and vagina: Narrowing down the number of candidate genes for Mayer-Rokitansky-Küster-Hauser syndrome. *Hum Genet.* 2021 Apr;140(4):667–80.
- Duncan PA, Shapiro LR, Stangel JJ, Klein RM, Addonizio JC. The MURCS association: Müllerian duct aplasia, renal aplasia, and cervicothoracic somite dysplasia. *J Pediatr.* 1979;95(3):399–402.
- Oppelt P, Renner SP, Kellermann A, Brucker S, Hauser GA, Ludwig KS, et al. Clinical aspects of Mayer-Rokitansky-Kuester-Hauser syndrome: Recommendations for clinical diagnosis and staging. *Hum Reprod.* 2006 Mar;21(3):792–7.
- Beski S, Gorgy A, Venkat G, Craft IL, Edmonds K. Gestational surrogacy: A feasible option for patients with Rokitansky syndrome. *Hum Reprod.* 2000;15(11):2326–8.
- Jones BP, Saso S, Bracewell-Milnes T, Thum M-Y, Nicopoulos J, Diaz-Garcia C, et al. Human uterine transplantation: A review of outcomes from the first 45 cases. *BJOG.* 2019 Oct;126(11):1310–9.
- Raziel A, Friedler S, Gidoni Y, Ben Ami I, Strassburger D, Ron-El R. Surrogate in vitro fertilization outcome in typical and atypical forms of Mayer-Rokitansky-Kuster-Hauser syndrome. *Hum Reprod.* 2012;27(1):126–30.
- Petrozza JC, Gray MR, Davis AJ, Reindollar RH. Congenital absence of the uterus and vagina is not commonly transmitted as a dominant genetic trait: Outcomes of surrogate pregnancies. *Fertil Steril.* 1997;67(2):387–9.
- Practice Committee of the American Society for Reproductive Medicine and Practice Committee of the Society for Assisted Reproductive Technology. Recommendations for practices utilizing gestational carriers: A committee opinion. *Fertil Steril.* 2017 Feb;107(2):e3–10.
- Fischer S, Gillman I. Surrogate motherhood: Attachment, attitudes and social support. *Psychiatry.* 1991;54:13–20.
- Jones BP, Ranaei-Zamani N, Vali S, Williams N, Saso S, Thum M-Y, et al. Options for acquiring motherhood in absolute uterine factor infertility; Adoption, surrogacy and uterine transplantation. *Obstet Gynaecol.* 2021 Apr;23(2):138–47.
- Kim HH. Selecting the optimal gestational carrier: Medical, reproductive, and ethical considerations. *Fertil Steril.* 2020;113(5):892–6.
- Consideration of the gestational carrier: An ethics committee opinion. *Fertil Steril.* 2018 Nov;110(6):1017–21.
- PR B. A Textbook of In Vitro Fertilization and Assisted Reproduction. London, UK and New York, NY: Taylor and Francis; 2005, pp. 393–404.
- Mizrachi Y, Horowitz E, Farhi J, Raziel A, Weissman A. Ovarian stimulation for freeze-all IVF cycles: A systematic review. *Hum Reprod Update.* 26(1):118–35.

31. Namath A, Jahandideh S, Devine K, O'Brien JE, Stillman RJ. Gestational carrier pregnancy outcomes from frozen embryo transfer depending on the number of embryos transferred and pre-implantation genetic testing: A retrospective analysis. *Fertil Steril.* 2021;115(6):1471–7.
32. Attawet J, Wang AY, Farquhar CM, Jordan V, Li Z, Sullivan EA. Pregnancy and birth outcomes of single versus multiple embryo transfer in gestational surrogacy arrangements: A systematic review and meta-analysis. *Hum Fertil (Camb).* 2020 Jul;1–11.
33. Utian WH, Goldfarb JM, Kiwi R, Sheean LA, Auld H, Lisbona H. Preliminary experience with in vitro fertilization-surrogate gestational pregnancy. *Fertil Steril.* 1989;52(4):633–8.
34. Meniru GI, Craft IL. Experience with gestational surrogacy as a treatment for sterility resulting from hysterectomy. *Hum Reprod.* 1997;12(1):51–4.
35. Corson SL, Kelly M, Braverman AM, English ME. Gestational carrier pregnancy. *Fertil Steril.* 1998;69(4):670–4.
36. Goldfarb JM, Austin C, Peskin B, Lisbona H, Desai N, de Mola JR. Fifteen years experience with an in-vitro fertilization surrogate gestational pregnancy programme. *Hum Reprod.* 2000;15(5):1075–8.
37. Brinsden PR, Appleton TC, Murray E, Hussein M, Akagbosu F, Marcus SF. Treatment by in vitro fertilisation with surrogacy: Experience of one British centre. *BMJ.* 2000;320(7239):924–8.
38. Attawet J, Wang A, Li Z, Johnson L, Hammarberg K, Sullivan E. Cumulative live birth rates among gestational surrogates in altruistic surrogacy arrangements. *Hum Fertil (Camb).* 2020 Jul;1–8.
39. Wang AY, Dill SK, Bowman M, Sullivan EA. Gestational surrogacy in Australia 2004–2011: Treatment, pregnancy and birth outcomes. *Aust N Z J Obstet Gynaecol.* 2016;56(3):255–9.
40. Swanson K, Letourneau JM, Kuppermann M, Einerson BD. Obstetric morbidity in gestational carrier pregnancies: A population-based study. *J Assist Reprod Genet.* 2021;38(1):177–83.
41. Söderström-Anttila V, Wennerholm U-B, Loft A, Pinborg A, Aittomäki K, Romundstad LB, et al. Surrogacy: Outcomes for surrogate mothers, children and the resulting families—a systematic review. *Hum Reprod Update.* 2016;22(2):260–76.
42. Tsutsumi R, Fujimoto A, Osuga Y, Ooi N, Takemura Y, Koizumi M, et al. Singleton pregnancy outcomes after assisted and non-assisted reproductive technology in infertile patients. *Reprod Med Biol.* 2012 Jul;11(3):149–53.
43. McCormick RA. Surrogacy: A catholic perspective. *Creighton Law Rev.* 1992;25(5):1617–25.
44. Schenker JG. Assisted reproduction practice in Europe: Legal and ethical aspects. *Hum Reprod Update.* 1997;3(2):173–84.
45. Benshushan A, Schenker JG. Legitimizing surrogacy in Israel. *Hum Reprod.* 1997;12(8):1832–4.
46. Husain FA. Reproductive issues from the Islamic perspective. *Hum Fertil (Camb).* 2000;3(2):124–8.
47. Jones HWJ, Cooke I, Kempers R, Brinsden P, Saunders D. International federation of fertility societies surveillance 2010: Preface. *Fertil Steril.* 2011;95:491.
48. Siddle N, Sarrel P, Whitehead M. The effect of hysterectomy on the age at ovarian failure: Identification of a subgroup of women with premature loss of ovarian function and literature review. *Fertil Steril.* 1987;47(1):94–100.
49. Golombok S, Murray C, Jadva V, MacCallum F, Lycett E. Families created through surrogacy arrangements: Parent-child relationships in the 1st year of life. *Dev Psychol.* 2004;40(3):400–11.
50. Crockin SL. Growing families in a shrinking world: Legal and ethical challenges in cross-border surrogacy. *Reprod Biomed Online.* 2013;27(6):733–41.
51. Swain ME, Rogerson CJ. Addressing legal issues in cross-border gestational surrogacy: Current topics and trends. *Fertil Steril.* 2021;115(2):268–73.
52. Practice Committee of the American Society for Reproductive Medicine and Practice Committee of the Society for Assisted Reproductive Technology. Recommendations for practices using gestational carriers: A committee opinion. *Fertil Steril.* 2022;118(1):65–74.

PATIENT SUPPORT IN THE ASSISTED REPRODUCTION TECHNOLOGY PROGRAMME

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Overview

Reproduction is considered the most basic of human needs, propelled by powerful biological and psychological drives. When the ability to reproduce is thwarted, a crisis ensues—the life crisis of infertility. The psychological crisis of infertility has been well documented in the literature. It is considered an emotionally difficult experience that impacts on all aspects of a couple's or an individual's life: relationships with others, life goals, social roles, self-image, self-confidence, and sexuality, to name a few [1]. The losses associated with infertility are multifaceted, including the loss of hopes, dreams, future plans, marital satisfaction, self-esteem, sense of control, belief in the fairness of life, health, and well-being, and, most important, the “dream child” [2]. Further, these losses evoke feelings of grief—shock, disbelief, sadness, anger, guilt, blame, and depression—which occur in a repetitive and predictable process as patients move through medical diagnosis and treatment. It is through the experience and expression of emotions involved in the grieving process that the infertile couple moves towards an acceptance of their infertile state, engages in the exploration of alternative plans, and begins to move forward with their lives [3].

During the past 50 years, we have seen a shift from the psychogenic infertility model, in which demonstrable psychopathology was thought to play an aetiological role in infertility, to a psychological sequelae model, in which numerous psychological factors were considered the result of infertility [4]. In this concept, infertility is viewed as an emotionally difficult experience affecting all aspects of an individual's and a couple's life. Thus, emotional distress is a consequence and not a cause of infertility, as conceptualized previously. The application of a broader spectrum of theoretical approaches has led to a less-individualistic perspective and a more holistic approach to infertility. In this sense, the interactions among individuals/couples and social/medical components are considered and must be factored into medical treatment. These perspectives have also increased understanding of individual/couple differences and resilience, the impact of reproductive medical treatments, and the efficacy of therapeutic psychological interventions.

Research examining the psychosocial context of infertility burgeoned during this period. In a comprehensive review of the literature, Greil and associates [5] expanded on earlier work [6] by assessing research over the years to determine how it has changed, where methodological progress has been made, and what generalizations can be drawn about the experience of infertility. They note the change from viewing infertility as a medical condition with psychological consequences to placing infertility within a larger sociocultural construct that shapes the experience. Thus, the individual or couple must define their inability to have children as a problem and then decide what they will do about it.

This conceptualization supports the shift in terminology from “infertility” to “fertility” care, counselling, and treatment [7]. Reproductive medical practices have historically been heteronormative in approach as treatment has focused on cisgender/different sex/heterosexual couples seeking care for infertility. However, in recent years there has been a shift in reproductive medical care as an ever-increasing number of patients seek assistance to have a family and who may not technically be infertile—single people, LGBTQ+ couples [lesbian, gay, bisexual, transgender, queer, plus (to represent self-identifying member of this community who are not represented in the former words)], and those seeking to preserve their fertility.

There is increasing consensus among all reproductive medical organizations that patients seeking assistance need a “patient-centred” approach whereby an individual patient's needs and values are respected, responded to, and guide all clinical decisions. Optimal patient care will include ways to minimize patients' psychological distress, while providing effective clinical care in a positive environment [8]. With a patient-centred approach, the provision of routine psychosocial care can reduce distress related to medical procedures and the experience of infertility, as well as improve patient well-being and compliance with treatment. Taking an evidence-based method, the European Society of Human Reproduction and Embryology (ESHRE) published extensive guidelines for routine psychosocial care in assisted reproduction technology (ART) programmes [9], an approach supported in this chapter.

Stress and ART

ARTs, while opening up expanded opportunities for the treatment of fertility, have generated their own psychological challenges for patients. For many couples, ARTs are the last, best options for having a child, and are used after long months, and sometimes years, of treatment failure, often at tremendous emotional, physical, and financial cost. (For other patients noted earlier who are technically not infertile, ART provides the chance to have a baby never before dreamed possible, yet it can take time to get pregnant and as treatment progresses many of the same stressors may occur.) Patients entering ART programmes usually do so with the burden of grief and disappointment from infertility, seeming depressed, angry, tired, dependent, and anxious. Although emotionally depleted, couples are attracted to a technology that offers hope where, a few years ago, none existed. They find themselves drawn into a new emotional turbulence of contrasting feelings of hope and despair, which seem to be generated in part by the experience of the technology itself. The intensity and high-tech nature of ART create a stressful atmosphere, where the stakes are high and the chance of success may be relatively low. ART is a gamble, and, like gamblers, patients may have unrealistically high expectations of success or feel compelled to try

"just one more time," finding it difficult to end treatment without success. Of all infertility treatments, *in vitro* fertilization (IVF) is considered the most stressful [10], with most patients classifying it as "extremely" to "moderately" stressful [11].

While the relationship between stress and infertility is not fully understood, IVF failure is known to cause significant psychological distress [12]. After a failed cycle, almost all couples report acute depression [13], with elevated anxiety and anger levels persisting weeks later [14]. Despite the stressful consequences of infertility and ART, numerous studies report that the vast majority of patients are generally well adjusted [15–18]. In one of the most extensive reviews of scientifically rigorous research on the psychological effects of infertility, Stanton and Danoff-Burg concluded that the majority of infertile men and women are psychologically resilient and maintain adequate psychosocial functioning [19]. Boivin found little evidence that infertile patients, as a group, experience significant, long-term maladjustment on measures of anxiety, psychiatric disturbance, marital conflict, and sexual dysfunction, when compared with population norms [20]. Overall, this group reports marital adjustment in the normal range, and that the crisis of infertility may actually improve marital communication and emotional intimacy [21–24].

Gender differences and ART stress

The majority of studies on stress during ART are in women, and, overall, women react more intensely to infertility and ART than do men [25]. Prior to IVF, women report more anxiety and depression, less life satisfaction, lower self-esteem, and more anticipatory stress than their male partners [22]. During IVF, the intensity of a demanding treatment protocol—daily ultrasound monitoring, blood draws for hormone levels, injections, invasive procedures for oocyte retrieval, and embryo transfer—is frequently given by women as a cause of psychological distress [10]. If treatment fails, depression persists longer for women than for their partners, lasting up to six months [13, 14]. Years later, women will recall the stress of IVF as more stressful for them than for their partners, regardless of the success or failure of treatment.

The male experience with ART has been largely overlooked by researchers [26], despite growing research indicating men do feel complex emotional reactions to an infertility diagnosis, including depression, helplessness, and threats to masculine identity [27]. Whereas the intensity of emotional reactions to particular aspects of ART may differ between men and women, the types of reactions are the same, with both experiencing a significant increase in anxiety and depressive symptoms from pre- to post-treatment [22]. In addition, both men and women rank the relative stresses of each stage of IVF equally and tend to overestimate the chances of success of IVF in general, showing a high level of hopefulness in their own cases [13].

Men and women tend to cope differently with the stress of ART and infertility. As frequently noted, women are more expressive of feelings, and are more likely to seek emotional and social support during ART by informal activities such as talking to a spouse, family, and friends. In terms of the effects of coping post-IVF treatment, Hynes et al. found that women who used problem-focused coping had a higher level of well-being than those who used avoidance coping or social support [28]. Men, on the other hand, who are often action-oriented and solution-focused, frequently cope with infertility through greater involvement in work or sports-related activities. While men and women may have different coping strategies, the use and effectiveness of these

techniques may be influenced by the point in the infertility process and the existence of a gender-specific infertility diagnosis [29].

Gender differences may also be impacted by the perception of psychosocial support during ART [30]. Since the nature of ART treatment is focused on women, men can feel more isolated and less emotionally supported than their partners, especially by family and friends [27]. Increased distress may arise when infertile people do not get the emotional support they need during infertility treatment. Psychosocial support and intervention are equally beneficial for both men and women [27, 31] and thus are recommended as part of treatment. It is also important to note that similar differences in stress and coping may also occur in same-sex couples, particularly lesbian couples. The non-carrying partner may feel marginalized and left out of treatment focus or when there is a loss. Thus, it is crucial that clinics provide an inclusive treatment environment which is supportive of same-sex couples in their family-building efforts [32].

Levels of stress during ART cycles

While general assumptions may be made about stress levels during ART cycles, the experience for infertility patients will be personal and unique: each patient will experience the stress differently, based upon his or her own personality and life experiences. Newton et al. noted that stress has been conceptualized both as a stimulus or event (distressing circumstances outside the person) and as a response (internal disturbance) [25]. A contrasting approach describes stress as neither an event nor a response, but rather a combination of factors: the perceived meaning of the event and self-appraisal of the adequacy of coping resources [33]. Thus, it is not the stress itself but the perception of the stress that determines how ART patients experience and handle it.

The aspects of ART perceived to be stressful to patients are multifaceted and affect all parts of their life: marital, social, physical, emotional, financial, cultural, and religious. Time is stressful, both in the time commitment to an intense treatment that leads to disruption in family, work, and social activities, and, for some, in long waiting periods for IVF or third-party reproduction. ART stress impacts on the marital relationship with an emotionally laden experience, and, by removing the conjugal act for procreation, sexual intimacy is lost. Also, couples are stretched financially, paying for the high cost of ART treatment with a relatively low probability of success. Dealing with the medical staff and with the side effects or potential complications of medical treatment has its own stress: hot flushes, headaches, mood fluctuations, shots, sonograms, future health concerns, and decision-making about embryos and multiple pregnancies. Religious, social, cultural, and moral issues may also make ART cycles stressful, especially for those dealing with third-party reproduction, when these values are in conflict with the choice of treatment.

The first treatment cycle has been found to be the most stressful for patients, with high levels of confusion, bewilderment, and anxiety [10, 14]. This may be due to inexperience with the process, or possibly inadequate preparation of the patient by staff in terms of information and discussion of care. Slade et al. found that for couples attempting three cycles of IVF, distress diminished during the middle cycle but rose after they discovered that the intervention had not been successful, with the last cycle being as stressful as the first [14].

Within a treatment cycle, patients view IVF/ART as a series of stages that must be successfully completed before moving on

to the next phase of treatment: monitoring, oocyte retrieval, fertilization, embryo transfer, waiting period, and pregnancy test stages. The level of stress, anxiety, and anticipation rises with each stage, peaking during the waiting period. A number of studies have confirmed what clinicians know anecdotally: in order of perceived stress for patients, waiting to hear the outcome of the embryo transfer is the most stressful, followed by waiting to hear whether fertilization has occurred, and then the egg retrieval stage [11, 34]. Patients are aware of the importance of these key phases in the IVF process, and the uncertainty of the outcome is highly distressing.

Understandably, patients who are experiencing emotional distress from infertility will have their quality of life impacted. To identify these patients, several years ago an international effort was undertaken by the American Society for Reproductive Medicine (ASRM), the ESHRE, and Merck-Serono to develop a psychometric tool that would be reliable, cross-cultural, and easy to access and interpret. Published in 2011, the Fertility Quality of Life (FertiQoL) is able to measure treatment quality (interactions with staff and quality of information) and treatment tolerability (effects on mood and disruptions to daily life), and proves to be an invaluable tool for clinicians [35]. It is free of charge and completed online by patients, with results sent to the clinician. FertiQoL is available in 46 languages, with more being developed, and takes about 10–15 minutes to complete (www.fertiqol.org).

Patient support diversity considerations in ART

Culture, race, ethnicity, sexual orientation, and gender identification can influence the experience of infertility and/or fertility treatment. These aspects may create distress, as well as generate various barriers that influence access to care. In recent years, we have begun to understand the implications of diversity factors in patient care and access to treatment. These factors influence the provision of patient support services and the importance of recognizing variations in patient needs.

Racial and ethnic differences can impact the care received due to institutional racism and discrimination that may exist within medicine which impacts diagnosis, care, and treatment outcomes [36]. All these differences may influence how the person understands and experiences infertility [37]. Racial and ethnic minority barriers can include stigmatization of fertility care, lack of fertility knowledge, language barriers, cultural stigma, discrimination, and a lack of trust in the medical system [38]. Among the LGBTQ+ population, barriers can include lack of services for this population, heteronormative and cisnormative care, psychological distress and triggering situations (e.g. patient examination of body parts), and stigmatization and discrimination [39]. These marginalized populations may be less likely to seek care because of fears around how they will be treated, or they may drop out of care if a perceived difficulty occurs.

Providers should be aware of potential disparities in fertility literacy with delivery of care. For example, in one recent US study, Hispanic participants were less likely to understand smoking-related harm to fertility, African Americans better understood the implications of sexually transmitted disease on fertility, and Asian respondents indicated a greater understanding of menstrual irregularities and infertility, as compared to Caucasians [37]. In providing patient support, the treatment team must be mindful of possible patient discrepancies in knowledge and understanding of their fertility.

Systemic changes to mitigate provider bias, increase fertility literacy, and increase quality research can help to address these disparities on a macro level [36]. On a clinic level, providers should be aware of pictures displayed, staff members represented, and language utilized [40]. To help providers avoid unintentional stereotyping or relying on assumptions, an open-ended approach to “cultural humility” is recommended. This concept involves understanding culture as an ongoing process of recognizing the complexities of various patient identities and experiences, with similarities and differences that can never be generalized to all people of all cultures [38].

Changes to patient support as a result of the pandemic

COVID-19 has been transformational in the way patient care is delivered. When the world shut down as the pandemic spread, a new need arose to function while limiting in-person contact. As people began to have more time at home and not travel, this freedom also allowed patients time and space to pursue fertility treatment. A drastic shift occurred from doing almost everything in person to providing almost all support by telehealth video technology.

Mental health implications of COVID-19 have been apparent with an increased prevalence of anxiety and depression growing across all populations by about 25% [41]. Thus, the need for patient support has grown. While fertility treatment temporarily halted at the beginning of the pandemic, mental health implications rose and the need for support services expanded. For infertility patients at one New England clinic, the top-rated stressor continued to be identified as their infertility, even well beyond the fears and stressors created by COVID-19 [42]. Once pregnancy was achieved after infertility, there was a shift in psychological burden to how COVID-19 would impact a pregnancy.

The use of technology and virtual visits has increased the demand for much needed and requested mental health care. The ability to provide fertility telemental health services has allowed patients to receive supportive care who previously may not have been able to take the time away from work due to multiple medical appointments. In the authors' clinical practice, the result has been a dramatic increase in patient requests for counselling and support services during their fertility journey, as a telehealth appointment takes less time off from work or home responsibilities than an in-office meeting.

Methods

Who provides patient support services in ART?

Given the host of research on the emotional consequences of infertility and on the distressing nature of ART, it is clear that patients need psychological support as an integrated part of the medical treatment process. Technology has become more complex, and so have the psychological, social, and ethical issues related to treatment, which challenges the resources of staff and patients. As a result of technological advances in ART and the recognition of the psychosocial issues and demands facing infertile patients, mental health professionals have become increasingly important members of the reproductive medical team. The specialization of “fertility counselling” has emerged internationally, combining the fields of reproductive health psychology and reproductive medicine, for mental health professionals including

social workers, psychologists, psychiatrists, marriage and family therapists, counsellors, and psychiatric nurses [7].

Fertility counsellors serve as a resource to patients and staff by providing specialized psychological services that support and enhance quality care. For example, the complex medical and psychological issues in third-party reproduction have psychosocial and legal implications that must be assessed carefully, and warrant involvement of a qualified mental health professional experienced in fertility counselling [43]. In addition, the psychosocial impact on the offspring created by ART needs to be considered, and assistance given to families dealing with these issues pre- and post-treatment.

Nevertheless, the responsibility for patient support in the ART programme is the duty of all staff members, not just the domain of nurses or fertility counsellors [9]. Interactions with each staff member, from administrative staff to physician, influence a patient's perception of care and, in turn, his or her stress level. Sensitivity, warmth, patience, and responsiveness create an environment of support. Also, the general clinic routine and ambience reflect support and respect of patients when it is provided in an efficient, organized, clean, uncrowded, and aesthetically pleasing atmosphere. All staff need to be sensitive to and knowledgeable about the psychological needs and stresses of ART patients [8]. While the primary focus of physicians, nurses, laboratory scientists, and other healthcare staff is the medical diagnosis and treatment of infertility, it must also entail "treating the patient, not the disease" [9].

Types of ART support services

ART patient support services can be generalized into overall clinic administration and environment to specialized services that need to be provided by a mental health professional who is trained and experienced as a fertility counsellor [43]. For the purpose of this chapter, while specialized services provided by a fertility counsellor are described, a detailed explanation of methodology is not addressed. (For further reference on this topic, the reader is directed to *Fertility Counseling: Clinical Guide* and *Fertility Counseling: Case Studies* [44].) Moving from specific to general, the methods of providing patient support services can be categorized as follows:

1. Psycho-education and implications counselling
2. Psychological assessment and preparation
3. Therapeutic counselling
4. Information and education
5. Technology and digital interventions
6. Clinical administration

Psycho-education and implications counselling

Psycho-education and implications counselling for participants using ART often vary from programme to programme, with the purpose often debated: should it be "mandatory" or "voluntary"? Is it "counselling" and/or "evaluation"? Currently, there are only a handful of jurisdictions that require counselling prior to ART treatment [45].

While the Human Fertilisation and Embryology Authority (HFEA), which regulates assisted reproduction in the United Kingdom, has stipulated that psychosocial counselling must be offered to patients seeking IVF or donor gametes [46], one study found that fewer than 25% of patients took up the suggestion [47]. In the United States, recommendations and guidelines for the

provision of psychological services to ART participants are voluntary [48], and the decision concerning which patients should be screened or counselled, and for what procedures, is left to each individual fertility practice. Thus, available guidelines for assessment and evaluation are usually tailored to the specific requirements or preferences of a particular programme. Whether a clinic adopts formal or recommended guidelines or chooses to develop its own, the programme's policy regarding fertility counselling, screening, exclusion criteria, and so on should be clearly defined for the protection of the medical team, the fertility counsellor, and patients [49].

Within the authors' programme, all intended parents/recipients of non-identified donor eggs, sperm, and embryos, and any parent(s) using a gestational carrier/surrogate, are required to see a fertility counsellor. The psycho-educational counselling usually take place in one counselling session. Reading materials and support resources are provided, and issues related to raising children conceived through third-party reproduction are discussed.

Psychological screening and preparation

Notwithstanding the voluntary nature of counselling ART participants, it has become the standard of care to require psychological screening and psycho-educational preparation of gamete donors and surrogate carriers by experienced fertility counsellors. In most programmes in the United States, the assessment usually involves both psychological testing of the donor/carrier, with the Minnesota Multiphasic Personality Inventory-2 (MMPI-2) [50, 51] or the Personality Assessment Inventory (PAI) [52], and clinical interviews with the donor/carrier and, when applicable, the partner. Assessment and counselling of intended parents of donor gametes are also strongly recommended or required by many programmes, especially when the donor/carrier is known or related. Other situations where programmes may require screening involve patients undergoing IVF who are considered psychologically or physically vulnerable and previous IVF patients donating frozen embryos.

The established protocol for psychological screening of donors and carriers and intended parents within the authors' programme includes the following:

1. Psychological screening of all non-identified oocyte donors is mandatory. Psychological testing (MMPI-2 or PAI) is administered and then scored and interpreted. Part of the extended interview includes only the donor with the other part including the donor and partner together. These are conducted with a fertility counsellor to assess psychological functioning, as well as to discuss the process, motivations, and implications of gamete donation.
2. All known donors (sperm, egg, embryo) or gestational carriers and the intended parents are required to undergo psycho-educational counselling and screening, which includes administering the MMPI-2 or PAI to the donor and gestational carrier. Clinical interviews are held with the donor or carrier and patient separately, including their partners, and a joint "group" session with all parties together is conducted to discuss how they will deal with issues in known donation or the surrogacy process. Legal consultation and contracts are also strongly recommended for known donors and required for gestational carriers.
3. Assessment and counselling of any fertility patient is required when the physician is concerned about psychological vulnerability or marital instability, or if a situation

is presented to our internal ethics committee where additional psychosocial information is needed before a decision about treatment can be made.

Our fertility counselling staff follow the criteria established for acceptance or rejection of participants in the recommended ASRM practice guidelines for gamete donors and gestational surrogates [48, 53]. When a recommendation to withhold or postpone treatment is made by the fertility counsellor, a team discussion takes place so that a decision is made by team consensus, rather than one member (usually the physician or the fertility counsellor) being seen by the patient as the "gatekeeper." It is useful to view and interpret these recommendations to the patient as protection of the parties involved rather than rejection, since it is the first responsibility of all healthcare providers to "do no harm."

Therapeutic counselling

Another aspect of patient support services involves intervention and treatment for the consequences of infertility, or for underlying mental disturbances that could affect medical treatment. Treatment modalities of individual, couple, and group counselling provide an opportunity to assist patients in understanding and handling: the emotional sequelae of infertility; identifying and developing a coping mechanism to deal with treatment; managing the effects of infertility and psychosocial history on interpersonal functioning (anxiety, depression, etc.); the impact on marital, sexual, and social relationships; the implications of ART treatment; decision-making on treatment options; alternative family building; pregnancy and parenting following treatment; ending treatment; and building a life after infertility. Group counselling has been shown to be a highly effective, cost-efficient intervention for producing positive change when education and skills training (e.g. relaxation techniques) are emphasized [31].

ART programmes may provide psychological assessment and therapeutic counselling services through a fertility counsellor on the staff (an employee) or on-site (an independent contractor) or may choose to refer to a qualified mental health professional who works independently of the clinic. Guidelines for when to refer patients to psychological support and assistance are displayed in Table 73.1.

Supportive counselling

Supportive counselling can be provided as a way to help patients with information gathering and decision-making while going through fertility treatment. Supportive counselling involves reproductive healthcare providers giving both advice (counsel) and comfort (console) to their patients. Although nurses often assume primary responsibility for patient support, it is the job of every member of the team to be empathic and sensitive to patients' needs. Services combined with psychoeducational counselling may include the following:

1. A pre-IVF preparation session with a fertility counsellor as part of the treatment package. This session can help to address expectations, coping mechanisms and counselling resources, and treatment decisions, including disposition of embryos.
2. Monthly support groups addressing a variety of topics for those with general infertility (non-ART); IVF participants; patients considering or using donor gametes; secondary infertility; miscarriage; LGBTQ+; single persons pursuing solo parenting; black, Indigenous, People of Colour

TABLE 73.1 Situations in Which Patients Are Referred to a Fertility Counsellor

The following situations serve as guidelines for referring patients to psychological counselling, screening, and/or intervention:

- The use or consideration of third-party reproduction
- Untreated psychiatric illness (past or present)
- History of pregnancy complications or loss
- Significant physical illness (past or present)
- Untreated sexual or physical abuse (past or present)
- Active chemical abuse or dependency
- Marital instability or chaotic social functioning

Symptoms

Referral to a mental health professional should also be considered when there is a change in current mental status and/or exacerbation of symptoms that are affecting normal functioning and relationships, including:

- Depression or persistent sadness and tearfulness
- High levels of anxiety or agitation
- Increased mood swings
- Obsessive-compulsive behaviours
- Strained interpersonal relationships
- Social isolation
- Loss of interest in usual activities
- Diminished ability to accomplish tasks
- Difficulty concentrating or remembering
- Difficulty making decisions
- Change in appetite, weight, or sleep patterns
- Increased use of drugs or alcohol
- Persistent feelings of pessimism, guilt, or worthlessness
- Persistent feelings of bitterness or anger
- Thoughts of or reference to death or suicide

(BIPOC) and infertility; and pregnancy after infertility. These groups may be open-ended, of no cost to patients, and run by a fertility counsellor and, if needed, a nurse.

3. A monthly discussion series on infertility topics identified through a patient survey, such as adoption, donor issues, staff-patient communication, drug side effects, dealing with family and friends, decision-making, marriage enhancement, and when to end treatment. These informal groups are facilitated by a fertility counsellor, physician, nurse, and/or an invited guest from the community who is knowledgeable on the subject.
4. Stress management and relaxation classes taught by a fertility counsellor and/or a nurse. Relaxation and guided imagery digital audio recordings may also available to patients for use before, during, and after retrieval and transfer.
5. Referral resources within the community for patients who request alternative approaches to help with quality of life during infertility, such as mind-body programmes, yoga classes, acupuncture, homeopathy, and weight management, can help to support a holistic approach.
6. Providing a network for patient-to-patient contact about aspects of treatment to support in making difficult decisions or digest and process information. Well-adjusted patients who have been through a procedure or have a specific diagnosis volunteer or are asked by a staff member if they would be willing to speak one-on-one with other

patients who request this contact. Common requests for contact are situations where patients have had a child via donor gametes, or who have undergone selective reduction or carried multiple pregnancies.

7. Providing current information about local and national infertility support groups (e.g. RESOLVE, Inc.), such as monthly updates on meetings, support groups, living room sessions, telephone counselling, newsletters, and articles.

Information and education

Probably the most far-reaching opportunity for ART support is through patients' easy access to written information and education about the medical and psychological aspects of infertility. Patients rely heavily on the educational materials that document the processes and procedures of ART, and search out information at the clinic, through the media (TV, magazines, books, etc.), and on the internet. One study found that patients identified informational materials as their primary source of support, after talking with a spouse, family, or friends [54].

Any information and treatment packets sent out to new patients should include material on the emotional aspects of infertility and on support resources available through the clinic, in the community, and via the internet. A clinic's website is also an important source of support information, and could connect to other internet resources, such as RESOLVE, for easy patient access. Examples of information and education support services include the following:

1. Online, interactive webcasts (webinars) on medical and psychosocial topics of infertility (i.e. preparing for IVF, deciding on ovum donation, miscarriage, etc.). These webinars are live and allow patients to ask questions, which are then archived on the clinic website for patients to access and review at a later time.
2. IVF and donor egg intended parents webinars for new patients beginning a cycle. Presentations can be made by a member of each treatment team—physician, embryology/laboratory, nurse, and/or fertility counsellor—and the administrative/finance office, who discuss protocols and processes, describe treatment services, and answer questions.
3. Ready access to pamphlets, articles, and written materials on the medical and emotional aspects of infertility, which are displayed in patient waiting areas and on the clinic website. Ample supplies of these materials are available in the nursing, physician, and fertility counselling offices, as well as with administrative staff. For example, billing staff found that as patients were checking out from office visits they often talked about their stresses and being able to give patients flyers on clinic support services or educational pamphlets was greatly appreciated.
4. A “fact sheet” of resources for patients with names, telephone numbers, and internet websites about clinic and community support services relating to infertility, endometriosis, primary ovarian insufficiency, polycystic ovary syndrome, adoption, pregnancy, pregnancy loss or termination, multiple gestation and parenting, and single parenting.
5. One-page “tip sheets” on topics that offer suggestions about coping with the emotional aspects of infertility (IVF, marital relationships, etc.) and “summary sheets” on medical treatments/procedures. Patient information “fact sheets”

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are also available through the ASRM's website (<https://www.reproductivefacts.org/news-and-publications/patient-fact-sheets-and-booklets/>) and can easily be downloaded and given to patients. These summary sheets are especially helpful, as the volume of information given to patients may be overwhelming, and research has shown that patients retain only a small portion of information given to them verbally.

6. A patient lending library of infertility-related books, videos, and audiotapes of instruction and information ranging from topics on sexual dysfunction and adoption to medical diagnosis and treatment of infertility.
7. Resources that can be accessed or downloaded from the clinic's website. These may include blogs and articles written by staff members on psychological and medical aspects of treatment, an “ask the expert” column for patients to write in questions, and online webcasts to present information on treatment programmes and psychosocial issues of infertility.

Technology and digital intervention

Digital technology has become a powerful source of information, education, and support for patients. There is increasing evidence that the internet can provide an effective intervention in helping patients manage the distress of infertility [55] and receiving direction from the medical staff on reliable internet sites for information is needed [56]. Social media, such as Facebook and Instagram, may become resources for support, interaction, and information for infertile patients when managed by the clinic, as well as providing a marketing tool for the practice. Multimedia methods, such as digital audio, video, interactive tasks, and personalized feedback, can serve as an effective psychosocial intervention [57] and should be considered by clinics as resources for patients. Finally, providing internet access to personal health records and medical data improves patient empowerment and satisfaction with care [58, 59].

The widening access to technology has produced another growing way to support patients as they go through treatment and beyond. More and more are actively using social media, online supports (i.e. blogs, podcasts), and apps as a way to find support. (However, social media can also be a source of great distress as patients compare themselves to others posting their pregnancy and birth successes.) These resources are low-cost and can be anonymous, providing a resource to those who might not utilize supportive counselling. The use of evidence-based and patient-centred fertility online support interventions in relation to specific issues, such as gender identification, sexual orientation, and ethnicity, can provide increased engagement [60]. Digital interventions can include education, self-help, peer and professionally lead support groups, and counselling, and may be used to provide emotional support, monitor quality of care, and provide patient-provider communication [61]. A few suggestions for ways to incorporate the use of digital intervention include the following:

1. The clinic can provide staff-monitored peer-to-peer social media sites, such as a Facebook group, specifically for the clinic and the type of treatment being utilized. This allows patients to provide information and support to each other.
2. Telehealth support groups led by a fertility counsellor to address topics such as general infertility, miscarriage, pregnancy after infertility, donor conception, single person pursuing independent parenting, LGBTQ+ using donor

conception, infertility among BIPOC, etc. Providing these support groups virtually allows for people in a variety of areas to have access.

3. Use of online medical portals to have easier access for contacting the treatment team with questions, scheduling appointments, or gaining access to reports of treatment results.
4. Offer a list of resources for digital apps (e.g. relaxation and guided meditation, fertility and cycle trackers, IVF trackers) and podcasts and Instagram accounts (e.g. both professional and personal accounts and information around infertility, reproductive health, and donor conception) which address various aspects of fertility treatment, care, education, and psychological care.
5. A programme-monitored, voluntary registry and forum for those who have used gamete donation to potentially connect to families who have used the same donor.

Clinic administration

The manner in which an ART programme is administered, along with the physical environment of the clinic, affects both patient stress levels and their perception of support. An aesthetically pleasing, clean, well-maintained office staffed by friendly, professionally dressed, well-trained people goes a long way in communicating an impression of professional competence, caring, and confidence. One study found that patient satisfaction can be improved with organizational shifts, when the patient was assigned a primary physician as well as being seen by a fertility-trained nurse [59]. Ways in which the authors' programme provides support through clinic administration include the following:

1. Private patient sitting areas and groupings within the waiting room, with access to reading materials, water, telephones, and restrooms. (If a clinic shares space with an obstetrics and gynaecology department, sensitivity needs to be considered and reasonable efforts made to separate pregnant patients and small children from infertility patients by adjusting appointments/schedules and/or seating arrangements.)
2. Private rooms where nurses or other clinical staff can instruct or consult with patients.
3. Private sections where billing and scheduling issues can be discussed by administrative staff with patients in a confidential manner.
4. A quiet, secure "donor room" for men to give semen samples, with erotic magazines/materials, a video player, and a comfortable chair or bed.
5. Private recovery areas after egg retrieval and embryo transfer with safe places to store belongings, a television/video player or music, and a comfortable chair for husbands.
6. Soothing, calming background music piped throughout the office.
7. An annual or biannual "baby party" for patients to come back with their children and celebrate with staff.
8. Miscarriage/pregnancy loss cards sent by the clinical staff when it is learned that, after a patient has been discharged from care, a pregnancy has been lost.
9. Primary care nursing, where a patient is assigned to one nurse, facilitating better continuity and coordination of treatment.
10. A staff member "patient advocate/ombudsman," who patients may talk to when they perceive a problem with

their care, or other conflicts with the clinic that cannot be resolved.

11. Patient surveys, suggestion boxes, and written feedback which encourage open communication regarding satisfaction, thoughts on improving care or services, and constructive criticism.
12. In-service training of all staff on the emotional needs of infertility patients, communication skills, stress management techniques, and on strategies to deal with difficult, demanding patients.
13. Staff support offering confidential assistance, direction, and referral for personal problems and professional burnout by the fertility counselling staff or through an employee assistance programme. Ultimately, happy staff members are productive workers who give the best support and service to patients.
14. Use of forms that consider diverse populations. The forms should be careful not to use heteronormative or cisnormative terms or assumptions, in addition to being mindful of language utilized to be inclusive of different racial and ethnic groups.
15. Resources and access to provide language services if the patients' native language is not the language spoken at the clinic.

Results

Although most patients undergoing ART cycles are well adjusted and will cope adequately with the process, all will benefit from, and indeed need, emotional support during treatment. Numerous studies show that most patients believe psychosocial counselling is beneficial and that they would avail themselves of it were it offered during treatment [20, 62, 63]. While a minority of patients experience significant emotional distress and use formal counselling services, the vast majority of those who use formal counselling report having found it helpful [20].

The efficiency of psychosocial interventions impacting mental health issues (i.e. depression, anxiety, and distress) and pregnancy rates during infertility is still being debated. A number of meta-analyses and systematic reviews have been reported over the years with mixed results [31, 64]. However, a study by Frederiksen and associates on research published between 1978 and 2014 suggests that psychosocial interventions for couples during infertility treatment, in particular cognitive behavioural therapy, are effective at both reducing psychological distress and at improving pregnancy rates [65].

There is a growing body of research examining the burden treatment places on patients both physically and psychologically [8, 66]. While patient-centredness is increasingly considered to be fundamental to quality care, medical professionals often misjudge themselves in how their patients experience their care and interactions [67]. The result may be that patients discontinue treatment because the emotional burden is too great. Thus, factors within the patient, the clinic, and the medical treatment contribute to this decision, and interventions must be addressed on all levels [68].

This information, coupled with the high dropout rates in ART programmes, most likely due to psychological reasons [69–71], suggests that IVF programmes need to provide better and more comprehensive psychosocial support services. Studies have indicated that even when cost is not a factor in pursuing treatment, more than half of patients drop out of treatment before depleting

their entitled insurance benefits [72–74]. Cross-culturally, the most common reason given for treatment termination is psychological burden and distress [66, 72–76]. Providing integrated psychological support services may be an important step in diminishing a patient's depression and anxiety, lowering dropout rates, and possibly even increasing pregnancy rates—the goal of all fertility programmes [70, 77, 78]. It may also increase patients' overall sense of satisfaction with care, even when pregnancy is not achieved [79].

Simple strategies for managing patients can help a great deal [8, 68]. Olivius and colleagues [71] found that ease in contacting the clinic or clinician by telephone, seeing the same doctor during treatment, and receiving sufficient oral and written information about treatment and complications helped with patient distress. At the very least, written materials and educational resources on the medical and psychosocial aspects of infertility need to be readily available and given to patients by their programmes. Further, the more holistically a patient is handled—supported medically and emotionally—the more likely she/he is to be treatment compliant and satisfied with care, regardless of the outcome of treatment. In fact, the true mark of success of a programme may be in the ability of the team to help patients feel that they, the patients, have done their best when treatment has failed (see Table 73.2 for a summary of strategies for ART patient support).

Future direction

Reproductive medicine will continue to change as advancing technology presents increasingly complex options and choices for patients. As reproductive technology continues to advance and push the boundaries of social, psychological, religious, and ethical acceptance, the need for comprehensive support services for ART patients will continue to grow. Patients will request a more holistic approach to medical treatment, where their bodies and their emotions are treated with equal importance. The authors believe that ART patients, as educated consumers, will search for the most effective and comprehensive care programme, often choosing a practice on the basis of whether psychological support services are integrated into treatment. There will continue to be a growing need for the specialized clinical skills and services of mental health professionals trained in fertility counselling to provide this assistance to patients and staff. ART programmes that have the foresight to integrate comprehensive support services with specialized mental health professionals as part of the treatment team will succeed.

Conclusion

Infertility is an emotionally exhausting, psychologically demanding experience for patients and, at times, their caregivers. Since ART cycles are considered the most stressful of all infertility treatments, patients who undergo them need as much support psychologically as they do medically from their clinical team. Specialized support services are needed for the psychosocial preparation, assessment, and treatment of patients who are faced with the unique issues associated with and/or the consequences of assisted reproduction. Experienced mental health professionals trained in fertility counselling must provide these specialized psychological services as part of, or in close collaboration with, the treatment team [80]. Finally, patient support is the responsibility of all employees of an ART programme, and staff must be knowledgeable about and sensitive to the emotional needs of their patients.

TABLE 73.2 Strategies for Assisted Reproduction Technology Patient Support

Before

- Educational classes presented by each member of the treatment team on IVF
- Pre-treatment counselling session with a fertility counsellor
- Psychosocial preparation and assessment of gamete donors, intended parents, and surrogates with a fertility counsellor
- Extensive written materials available and distributed on the medical, emotional, and financial aspects of ART
- Educational videos and web-based support on the medical and emotional aspects of infertility and ART
- Support groups
- Stress management, relaxation, and guided imagery classes as well as information on phone apps
- Resource lists of community support services, including RESOLVE, Inc.

During

- Access to the fertility counsellors and other team members
- Telephone support with a primary care nurse
- If a patient has met with a fertility counsellor before starting the cycle, a brief visit in the OR on retrieval and/or transfer day
- Stress management, relaxation, and guided imagery classes and phone apps
- Computer-based technology, including a clinic website with resource materials and interactive social media (e.g. Facebook and Twitter), online educational webinars, written materials identifying reliable internet sites for information and support
- Support groups

After

- Psychosocial follow-up after a failed cycle or pregnancy loss
- Decision-making counselling regarding alternative therapies or ending treatment
- Counselling on alternative family building through adoption or third-party reproduction
- Counselling and support for the decision to remain child-free after infertility
- Counselling and preparation for multiple pregnancy, including selective reduction
- Counselling and follow-up for pregnancy after infertility, including support groups
- Counselling and follow-up for issues in parenting after infertility, including families created through donor gametes
- Support groups
- Patient feedback survey

Abbreviations: ART, assisted reproduction technology; IVF, *in vitro* fertilization; OR, operating room.

References

1. Menning BE. The emotional needs of infertile couples. *Fertil Steril*. 1980;34:313–9.
2. Mahlstedt PP. The psychological component of infertility. *Fertil Steril*. 1985;43:335–46.
3. Stanton AL, Dunkel-Schetter C. Psychological adjustment to infertility. In: Infertility: Perspectives from Stress and Coping Research. Stanton AL, Dunkel Schetter C (eds). New York, NY: Plenum Press, 1991, pp. 3–16.

4. Burns LH, Covington SN. Psychology of infertility. In: Infertility Counseling: A Comprehensive Handbook for Clinicians, 2nd ed. Covington SN, Burns LH (eds). New York, NY: Cambridge University Press, 2006, pp. 1–19.
5. Greil AL, Slauson-Blevins K, McQuillan J. The experience of infertility: A review of recent literature. *Soc Health Illn.* 2010;32: 140–62.
6. Greil A. Infertility and psychological distress: A critical review of the literature. *Soc Sci Med.* 1997;45:1679–704.
7. Covington SN (ed.). Fertility Counseling: Clinical Guide and Case Studies. London: Cambridge University Press, 2015.
8. Gameiro S, Boivin J, Domar A. Optimal in vitro fertilization in 2020 should reduce treatment burden and enhance care delivery for patients and staff. *Fertil Steril.* 2013;100:302–9.
9. Gameiro S, Boivin J, Dancet E, et al. ESHRE guideline: Routine psychosocial care in infertility and medical assisted reproduction—A guide for fertility staff. *Hum Reprod.* 2015;30:2476–85.
10. Boivin J, Griffiths E, Venetis CA. Emotional distress in infertile women and failure of assisted reproductive technologies: Meta-analysis of prospective psychosocial studies. *BMJ.* 2011;342:223.
11. Matthiesen SMS, Frederiksen Y, Ingerslev HJ, Zachariae R. Stress, distress, and outcome of assisted reproductive technology (ART): A meta-analysis. *Hum Reprod.* 2011;26:2763–76.
12. Pasch LA, Gregorich SE, Katz PK, Millstein SG, Nachtigall RD, Bleil ME, Adler NE. Psychological distress and in vitro fertilization outcome. *Fertility and Sterility.* 2012;98(2):459–464.
13. Holley SR, Pasch LA, Bleil ME, Gregorich S, Katz PK, Adler NE. Prevalence and predictors of major depressive disorder for fertility treatment patients and their partners. *Fertil Steril.* 2015;103:1332–9.
14. Slade P, Emery J, Lieberman BA. A prospective, longitudinal study of emotions and relationships in in vitro fertilization treatment. *Hum Reprod.* 1997;12:183–90.
15. Connolly KJ, Edelmann RJ, Cooke ID, Robson J. The impact of infertility on psychological functioning. *J Psychosom Res.* 1992;36:459–68.
16. Paulson JD, Haarmann BS, Salerno RL, Asmar P. An investigation of the relationship between emotional maladjustment and infertility. *Fertil Steril.* 1988;49:258–62.
17. Downey J, Husami N, Yingling S, et al. Mood disorders, psychiatric symptoms and distress in women presenting for infertility evaluation. *Fertil Steril.* 1989;52:425–32.
18. Edelmann RJ, Connolly KJ, Cooke ID, Robson J. Psychogenetic infertility: Some findings. *J Psychosom Obstet Gynecol.* 1991; 12:163–8.
19. Stanton AL, Danoff-Burg S. Selected issues in women's reproductive health: Psychological perspectives. In: The Psychology of Women's Health: Progress and Challenges in Research and Application. Stanton AL, Gallant SJ (eds). Washington, DC: American Psychological Association, 1996, pp. 261–305.
20. Boivin J. Is there too much emphasis on psychosocial counseling for infertile patients? *J Assist Reprod Genet.* 1997;14:184–6.
21. Freeman EW, Rickels K, Tausig J, et al. Emotional and psychosocial factors in follow-up of women after IVF-ET treatment. *Acta Obstet Gynecol Scand.* 1987;66:517–21.
22. Newton CR, Hearn MT, Yuzpe AA. Psychological assessment and follow-up after in vitro fertilization: Assessing the impact of failure. *Fertil Steril.* 1990;54:879–86.
23. Berg BJ, Wilson JF. Psychological functioning across stages of treatment for infertility. *J Behav Med.* 1991;14:11–26.
24. Lalos A, Lalos O, von Schoultz B. The psychosocial impact of infertility two years after completed surgical treatment. *Acta Obstet Gynecol Scand.* 1985;65:599–604.
25. Newton CR, Sherrard W, Glavac I. The Fertility Problem Inventory: Measuring perceived infertility-related stress. *Fertil Steril.* 1999;72:54–62.
26. Petok WD. Infertility counseling (or the lack thereof) of the forgotten male partner. *Fertility and Sterility.* 2015;104(2):260–6.
27. Peterson B, Petok WD. The male experience with fertility and counselling. In: Fertility Counseling: Clinical Guide, 2nd ed. Covington SN (ed.). London: Cambridge University, 2023, pp 153–161.
28. Hynes GJ, Callan VJ, Terry DJ, et al. The psychological well-being of infertile women after a failed IVF attempt: The effects of coping. *Br J Med Psychol.* 1992;65:269–78.
29. Petok WD. The psychology of gender-specific infertility diagnoses. In: Infertility Counseling: A Comprehensive Handbook for Clinicians, 2nd ed. Covington SN, Burns LH (eds.). New York, NY: Cambridge University Press, 2006, pp. 37–60.
30. Agostini F, Monti F, De Pascalis L, et al. Psychosocial support for infertile couples during assisted reproductive technology treatment. *Fertil Steril.* 2011;95:707–10.
31. Boivin J. A review of psychosocial interventions in infertility. *Soc Sci Med.* 2003;57:2325–41.
32. Holley S, Pasch L. Counseling lesbian, gay, bisexual, and queer fertility patients. In: Fertility Counseling: Clinical Guide, 2nd ed. Covington SN (ed.). London: Cambridge University Press, 2023, pp. 162–71.
33. Cohen SJ, Kessler RC, Underwood GL. Strategies for measuring stress in studies of psychiatric and physical disorders. In: Measuring Stress: A Guide for Health and Social Scientist. Cohen SJ, Kessler RC, Underwood GL (eds.). New York, NY: Oxford University Press, 1995, pp. 3–25.
34. Boivin J, Takefman J. Stress level across stages of in vitro fertilization in subsequently pregnant and non-pregnant women. *Fertil Steril.* 1995;64:802–10.
35. Boivin J, Takefman J, Braverman A. The Fertility Quality of Life (FertiQoL) tool: Development and general psychometric properties. *Fertil Steril.* 2011;96:409–15.
36. Jackson-Bey T, Morris J, Jasper E, Velez Edwards DR, Thornton K, Richard-Davis G, Comeaux Plowden T. Systematic review of racial and ethnic disparities in reproductive endocrinology and infertility: Where do we stand today? *Fertil Steril Rev.* 2021;2(3): 169–88.
37. Maher JY, Pal L, Illuzzi JL, Achon MN, Lundsberg LS. Racial & ethnic differences in reproductive knowledge & awareness among women in the U.S. *F S Rep.* 2022 April 3;(2 Suppl):46–54.
38. Kirubarajan A, Patel P, Leung S, Prethipan T, Sierra S. Barriers to fertility care for racial/ethnic minority groups: A qualitative systematic review. *Fertil Steril Rev.* 2021;2(2):150–9.
39. Kirubarajan A, Patel P, Leung S, Park B. Cultural competence in fertility care for lesbian, gay, bisexual, transgender, and queer people: A systematic review of patient and provider perspectives. *Fertil Steril.* 2021;115(5):1294–301.
40. Davoudian T, Covington L. Pregnancy and postpartum adjustment in fertility counseling. In: Fertility Counseling: Clinical Guide, 2nd ed. Covington SN (ed.). London: Cambridge University Press, 2023, pp. 234–244.
41. World Health Organization (WHO). Mental Health and COVID-19: Early evidence of the pandemic's impact. Scientific brief. 2 March 2022. <https://apps.who.int/iris/rest/bitstreams/1412184/retrieve>
42. Domar AD, Shah JS, Gompers A, Meyers AJ, Khodakham DR, Hacker MR, Penzias AS, Sakkas D, Toth TL, Vaughan DA. The psychological impact of the coronavirus disease 2019 pandemic on women who become pregnant after receiving treatment for infertility: A longitudinal study. *Fert Sertil Rev.* 2022;3(1):71–8.
43. Practice Committee and the Mental Health Professional Group of the American Society for Reproductive Medicine. Guidance on qualifications for fertility counselors: A committee opinion. *Fertil Steril.* 2021;115(6):1411–5.
44. Covington SN (ed.). Fertility Counseling: Clinical Guide, 2nd ed. and Fertility Counseling: Case Studies, 2nd ed. London: Cambridge University Press, 2023.
45. Blyth E. Guidelines for infertility counselling in different countries: Is there an emerging trend? *Hum Reprod.* 2012;27:2046–57.

46. Human Fertilisation and Embryology Authority. Code of Practice, 2nd ed. London: HFEA, 1995.
47. Hernon M, Harris CP, Elstein M, et al. Review of organized support network for infertility patients in licensed units in the UK. *Hum Reprod.* 1995;10:960–4.
48. Practice Committee of the American Society for Reproductive Medicine and the Practice Committee for the Society for Assisted Reproductive Technology. Guidance regarding gamete and embryo donation. *Fertil Steril.* 2021;115(6):1395–410.
49. Klock SC, Maier D. Guidelines for the provision of psychological evaluations for infertile patients at the university of Connecticut health center. *Fertil Steril.* 1991;56:680–5.
50. Klock SC, Covington SN. Minnesota Multiphasic personality inventory (MMPI-2) profiles in the assessment of ovum donors. *Fertil Steril.* 2010;94:1684–8.
51. Klock SC. Results of the minesota multiphasic personality inventory-2 among gestational surrogacy candidates. *Int J Gynecol Obstet.* 2015;130:257–60.
52. Sims JA, Thomas KM, Hopwood CJ, Chen SH, Pascale C. Psychometric properties and norms for the personality assessment inventory in egg donors and gestational carriers. *J Pers Assess.* 2013;95(5):495–9.
53. Practice Committee of the American Society for Reproductive Medicine and Practice Committee of the Society for Assisted Reproductive Technology. Recommendations for practices utilizing gestational carriers: A committee opinion. *Fertil Steril.* 2017;107(2):e3–e10.
54. Boivin J, Scanlan LC, Walker SM. Why are infertile patients not using psychosocial counselling? *Hum Reprod.* 1999;14:1384–91.
55. Cousineau TM, Green TC, Corsini E, et al. Online psychoeducational support for infertile women: A randomized controlled trial. *Hum Reprod.* 2008;23:554–66.
56. Kahlor L, Mackert M. Perceptions of infertility information and support sources among female patients who access the internet. *Fertil Steril.* 2009;91:83–90.
57. Cousineau TM, Lord SE, Seibring AR, et al. A multimedia psychosocial support program for couples receiving infertility treatment: A feasibility study. *Fertil Steril.* 2004;81:532–8.
58. Tuil W'S, Verhaak CM, Braat D, de Vries Robbe P, Kremer J. Empowering patients undergoing in vitro fertilization by providing internet access to medical data. *Fertil Steril.* 2007;88:361–8.
59. Van Emple I, Hermens R, Akkermans R, et al. Organization determinants of patient-centered fertility care: A multilevel analysis. *Fertil Steril.* 2011;95:513–9.
60. Grunberg PH, Dennis CL, Da Costa D, Zelkowitz P. Infertility patients' need and preferences for online peer support. *Reprod Biomed Soc Online.* 2018;6:80–9.
61. Hammarberg K, Schmidt L, Malling G, Koert E. Using technology to enhance communication in medically assisted reproductive care. In: Patient-Centered Assisted Reproduction: How to Integrate Exceptional Care with Cutting Edge Technology. Domar, AD, Sakkas, D, and Toth, TL (eds.). Cambridge, UK: Cambridge University Press, 2020, pp. 95–105.
62. Baram D, Tourtelot E, Muechler E, et al. Psychosocial adjustment following unsuccessful in vitro fertilization. *J Psychosom Obstet Gynecol.* 1988;9:181–90.
63. Mazure CM, Greenfeld DA. Psychological studies of in vitro fertilization/embryo transfer participants. *J In Vitro Fert Embryo Transf.* 1989;6:242–56.
64. Hammerli K, Hansjorg Z, Barth J. The efficacy of psychological interventions for infertile patients: A meta-analysis examining mental health and pregnancy rate. *Hum Reprod Update.* 2009;15:279–95.
65. Frederiksen Y, Farver-Vestergaard I, Skovgard NG, Ingerslev HJ, Zachariae R. Efficacy of psychosocial interventions for psychological and pregnancy outcomes in infertility women and men: A systematic review and meta-analysis. *BMJ Open.* 2015;5:e006592.
66. Gamerio S, Boivin J, Peronace LA, Verhaak CM. Why do patients discontinue treatment? A systemic review of reason and predictors of discontinuation in fertility treatment. *Hum Reprod Update.* 2012;18:652–69.
67. Aarts WM, Faber MJ, van Empel IWH, Scheenjes E, Nelen WLDM, Kremer JAM. Professionals' perceptions of the patients' experiences with fertility care. *Hum Reprod.* 2011;26:1119–27.
68. Boivin J, Domar AD, Shapiro DB, Wischmann T, Fauser BC, Verhaak CM. Tackling burden in ART: An integrated approach for medical staff. *Hum Reprod.* 2012;27:941–50.
69. Domar AD. Impact of psychological factors on dropout rates in insured infertility patients. *Fertil Steril.* 2004;81:271–3.
70. Penzias AS. When and why does the dream die? Or does it? *Fertil Steril.* 2004;81:274–5.
71. Olivius C, Friden B, Borg G, Bergh C. Why do couples discontinue in vitro fertilization treatment? A cohort study. *Fertil Steril.* 2004;81:258–61.
72. Smeenk JMJ, Verhaak CM, Stolwijk AM, Kremer JA, Braat DD. Reasons for dropout in an in vitro fertilization/intracytoplasmic sperm injection program. *Fertil Steril.* 2004;81:262–8.
73. Malcolm CE, Cumming DC. Follow-up of infertile couples who dropped out of a specialist fertility clinic. *Fertil Steril.* 2004;81:269–70.
74. Brandes M, van der Steen J, Bokdam S, et al. When and why do subfertile couples discontinue their fertility care? *Hum Reprod.* 2009;24:3127–35.
75. Domar AD, Smith Conboy L, Iannone M, Alper M. A prospective investigation into the reasons why insured United States patients drop out of in vitro fertilization treatment. *Fertil Steril.* 2010;94:1457–9.
76. Terzioglu F. Investigation into the effectiveness of counseling on assisted reproductive techniques in Turkey. *J Psychosom Obstet Gynaecol.* 2001;22:133–41.
77. Smeenk JMJ, Verhaak CM, Stolwijk AM, Kremer JAM, Braat DDM. Psychological interference in in vitro fertilization treatment. *Fertil Steril.* 2004;81:277.
78. Mourad SM, Nelen W, Akkermans R, et al. Determinants of patients' experiences and satisfaction with fertility care. *Fertil Steril.* 2010;94:1254–60.
79. Olivius C, Friden B, Borg G, Bergh C. Psychological aspects of discontinuation of in vitro fertilization treatment. *Fertil Steril.* 2004;81:276.
80. Covington SN, Adamson GD. Collaborative reproductive healthcare model. In: Fertility Counseling: Clinical Guide and Case Studies. Covington SN (ed.). Cambridge, UK: Cambridge University Press, 2015, pp. 1–32.

THE RELATIONSHIP BETWEEN STRESS AND IN VITRO FERTILIZATION OUTCOME

Alice D. Domar

Introduction

For thousands of years women have been facing the same biased assumption—infertility is their fault. And not only is it their fault, but their sadness/anger/distress could be a main cause. This belief has led to centuries of unsolicited advice, unproven remedies, and, in the current times, suggestions which reflect this bias: “you’re trying too hard, just relax,” “if you adopt, you’ll get pregnant,” “maybe you need to quit your job, you’re so anxious.”

So the main theme that women in an infertile couple have been hearing is that their mood state is entirely responsible for their lack of conception and if they would only cheer up, voila, pregnancy.

However, with the advent of better diagnostic technologies, in the past 50 or so years, the scientific community has gone almost entirely in the opposite direction. Currently, most cases of infertility are attributed to diagnoses in the female, the male, or both partners. Any psychological basis of infertility is dismissed. By most physicians. Strangely enough, however, many infertility patients continue to attribute stress as a cause or at least a contributor. Many patients, when faced with a negative pregnancy test after a treatment cycle, will ponder what might have contributed to that failure—was it their fight with their mother-in-law? The crazy deadline at work? Were they not hopeful enough? Were they feeling too overwhelmed and anxious about the injections?

So who is right? Is infertility entirely physiological and the mind or mood play no role, or can stress have an impact on treatment? Can stress have an impact on ART outcome?

The psychological impact of infertility

Research conducted over the past 20 years or so has, in fact, documented that individuals, both women and men, experiencing infertility do report very high levels of negative psychological symptoms. In a study at UCSF of 352 women and 274 men undergoing infertility treatment [1], 56% of women and 32% of men scored in the clinical range for depression, and 76% of women and 61% of men scored in the clinical range for anxiety. In a unique study where instead of asking patients to self-report symptoms of distress, which may not be necessarily effective in a patient population that may want to “fake good” so that their physician remains ignorant of their psychological status, a structured psychiatric interview was utilized [2]. The participants were assessed prior to being seen in an infertility clinic for the first time; 40% were diagnosed as having anxiety, depression, or both. It is likely that this number increased greatly as these women underwent a diagnostic workup and subsequent treatment.

In one literature review, the prevalence of psychiatric issues among individuals with infertility ranged from 25% to 60% [3]. Most reviews conclude that upwards of 50% of infertile women report significantly higher distress levels than fertile women.

Men in an infertile couple also report higher distress levels than in the fertile population but at a lower intensity and prevalence than women.

Thus, it is clear that infertility leads to significant levels of distress, in both men and women. But does stress cause infertility? And can stress impact the outcome of *in vitro* fertilization (IVF)?

The impact of distress on IVF outcome

There have been dozens of studies over the past 20 to 30 years investigating the impact of self-reported distress levels on IVF outcome. Some have shown a positive relationship, i.e. the higher the levels of distress, the lower the pregnancy rates, while others have shown no significant relationship.

A positive relationship

In one of the earliest studies on IVF patients, Smeenk et al. [4] prospectively psychologically assessed 291 women prior to starting downregulation medication with anxiety and depression scales. Even when controlling for medical factors, there was a significant relationship between the baseline psychological scores and pregnancy rates, with a slightly stronger relationship for a state of anxiety ($p = 0.01$) than depression ($p = 0.03$). The authors concluded that patients’ baseline psychological states were independently correlated to success rates and suggested that psychological interventions could improve pregnancy rates from IVF.

A subsequent study on 47 women scheduled to undergo their second IVF/ICSI cycle focused on episodic anxiety [5]. The women who reported high episodic anxiety were less likely to conceive, although a positive relationship between trait or state anxiety was not found.

Stressful life events are also positively correlated to poorer IVF outcomes. In a study of 809 women assessed prior to their IVF treatment, women who reported fewer non-infertility related negative life events were significantly more likely to conceive ($p = 0.02$), even after controlling for numerous factors including age, diagnosis, duration, and number of retrieved oocytes [6].

In a study of 81 female IVF patients, participants completed numerous psychological questionnaires prior to treatment [7]. The measures included basic psychological symptoms such as anxiety and depression, as well as narcissism and alexithymia, which is defined as the inability to relate to or describe one’s own emotions. Younger age and more alexithymic features were correlated to higher pregnancy rates. The authors attributed the positive impact of alexithymia due to the “operational” nature of IVF, for which alexithymic individuals may well cope better.

Similar results came from a study published in 2011 [8]. A total of 160 women undergoing IVF were assessed for biomedical and psychological factors prior to treatment. When controlling for factors such as age, oocyte number, and number of embryos transferred, infertility stress and nonspecific anxiety

were significantly negatively associated with pregnancy outcome. These authors also concluded that counselling interventions had the potential to improve treatment outcome.

In a study which included both self-report psychological questionnaires as well as the collection of serum norepinephrine and cortisol in 264 women undergoing IVF, the results were similar to the previous reports [9]. Women who had unsuccessful cycles had reported higher baseline levels of anxiety and depression when compared to the successful patients. Lower levels of norepinephrine and cortisol at the time of oocyte retrieval were also positively correlated with pregnancy.

In another study on 45 couples undergoing IVF, both self-report psychological questionnaires as well as cytokine levels were included [10]. Almost three-quarters of the participants reported elevated levels of negative psychological symptoms on the questionnaires. There was a lower likelihood of both pregnancy and live birth when participants reported higher levels of stress, and cytokine levels in both partners were correlated to IVF failure.

Finally, in the most recent study, 304 infertile women were prospectively studied prior to IVF [11]. A total of 80% had high depression scores, and their global stress scores and anxiety levels were negatively correlated with clinical pregnancy rates. These authors also strongly emphasized the need for "specific psychological interventions" to be made available to all IVF patients in an effort to increase pregnancy rates from IVF.

No relationship

In a study of 783 women undergoing their first IVF cycle, levels of anxiety and depression were assessed at pre-treatment baseline, and procedure anxiety the day before oocyte retrieval [12]. Neither anxiety nor depression were significantly related to pregnancy rate, and cycle cancellation was also not predicted by psychological factors.

Subsequently, in another study of 202 women who were starting their first IVF cycle and then followed for up to 18 months, pre-treatment levels of anxiety and depression did not significantly correlate with pregnancy rates [13]. The authors pointed out that their results indicated that psychological distress does not predict outcome, but that IVF failure does in fact predict psychological distress.

In a smaller study of 108 women about to undergo their first IVF cycle, psychological and physiological variables were included [14]. There were no relationships between any of the collected psychological variables (state anxiety, depression, and overall psychiatric symptoms) and outcomes, including the number of collected and fertilized oocytes, positive beta pregnancy rates, or live births.

A study on both psychological and physiological assessments in 485 women undergoing IVF also revealed no correlation with clinical pregnancy rates [15]. Psychological questionnaires as well as salivary cortisol levels were collected prior to cycle start. The associations between the stress measures and cycle outcome were adjusted for age, BMI, several lifestyle habits, and education.

Similarly, in a study of 142 women undergoing IVF, when controlling for diagnosis, age, and duration, there was no relationship between depression, stress, or anxiety and clinical pregnancy rate [16].

Finally, in the most recent study, which included 150 women who had an unsuccessful first IVF cycle, there were no significant correlations noted between pre-treatment psychological distress and clinical pregnancy rate [17]. However, it is notable in

this study that far fewer participants noted psychological symptoms than found in other comparable studies; only 14% reported depressive symptoms and 2.4% with symptoms of anxiety. Thus, this patient sample may well not be representative of IVF patients globally.

Conflicting results

In a small study of 22 women entering IVF treatment and 22 fertile controls, state of anxiety was assessed in the IVF patients prior to cycle start as well as blood tests in both groups [18]. The infertility patients scored significantly higher than the controls in suspicion, guilt, and hostility, but lower on somatic anxiety and indirect aggression. The infertility patients had significantly higher levels of prolactin and cortisol throughout their cycles. Baseline serum cortisol, prolactin, and FSH levels on day 3 did not correlate with pregnancy rates in the IVF patients, but there was a trend ($p < 0.06$) for higher state anxiety levels in the unsuccessful women, a value which could well have reached significance with a larger study group.

Finally, another study with somewhat conflicting results included 61 women about to start their first IVF cycle [19]. Each participant completed a battery of psychological questionnaires. Life purpose and negative emotions were *positively* associated with pregnancy rates while autonomy and stress were *negatively* associated, leading the authors to conclude that the relationship between psychological factors and IVF success may be more complicated than anticipated.

The meta-analyses

In a systematic review and meta-analysis on the relationship between psychological factors and ART outcome from 2011 [20], 31 prospective studies were included. There were "small, statistically significant pooled effect sizes" determined for state as well as trait anxiety and stress, and a trend for depression ($P = 0.06$). The authors concluded that there are small but significant correlations between distress and IVF outcome but noted the study heterogeneity.

To add to the confusion, two meta-analyses on the impact of emotional distress on IVF outcome, published the same year, came to directly opposite conclusions. One included 11 studies on 2202 patients and demonstrated that anxiety and depression were significantly associated with lower pregnancy rates [21]. The other meta-analysis included 4308 patients from 20 studies, and those authors concluded that symptoms of distress during treatment were *not* associated with pregnancy rates [22].

Physiological support for a relationship

There have been several studies which have investigated the role of hair cortisol as a marker and predictor of the impact of stress on IVF outcome. In the first study [23], 88 IVF patients about to undergo treatment provided hair samples, as hair sampling provides the systematic levels of cortisol from the prior three to six months. The hair cortisol levels significantly predicted clinical pregnancy rates ($p = 0.017$). This relationship remained significant when controlling for the accumulated salivary cortisol and accounted for 27% of the variance in pregnancy outcome.

In a subsequent study, 43 IVF patients had their hair cortisol concentration level (HCC) assessed prior to cycle start as well as post transfer, in addition to completing psychological questionnaires at both time points [24]. HCC at time 2 predicted 46% of positive pregnancy test variance. Women who conceived also had higher levels of resilience at time 2. There were significant

differences found between the successful versus unsuccessful patients in depression and resilience at time 2. The authors concluded with the recommendation that HCC could be used as a predictor of pregnancy in IVF patients.

Inconclusive physiological data

In a systematic review on the association between cortisol and IVF outcome, eight studies were determined to indicate a significant association [25]. Three of the studies reported a positive significant relationship, in that *higher* cortisol was associated with higher pregnancy rates, while five studies reported that *lower* cortisol levels were associated with a positive outcome. The researchers reported that the evidence which suggests a link between cortisol and IVF is inconclusive and attributed some of the confusion to methodological limitations of the research.

How to explain the contradictory findings?

As one can see from the previously cited research, there is a fair amount of data to support the hypothesis that stress in fact can have a negative impact on IVF outcomes. However, there is some solid contradictory evidence as well. How to explain the discrepancy? One of the main reasons is that almost all of this research has used self-report psychological questionnaires. And this can be problematic since patients may well “fake good” with self-report questionnaires. They may not want their physicians to know how distressed they really are, and/or they may be from a culture where one doesn’t disclose negative psychological symptoms. It is not unusual in this field for a highly distressed patient to produce a score of 0 on a questionnaire, both in a clinical and in a research setting.

Another complicating factor is the indirect impact of stress on fertility. Stress can impact lifestyle factors which effect fertility, such as smoking, exercise, lower libido, caffeine consumption, sleep, and eating behaviours. So it is possible that it is not the physiological impact of stress on fertility per se, but instead an indirect negative impact of increased harmful lifestyle behaviours.

There are other limitations to the interpretation of this research as well, mainly the variability of the different studies. Some of the studies assessed distress months prior to cycle start, some on the first day of gonadotropin administration, some a week later, some on the day of oocyte retrieval, etc.

Finally, it is common for IVF patients to feel a sense of optimism and hope prior to their first IVF cycle. Pregnancy rates for IVF far exceed prior treatment options and thus many patients don’t experience high levels of distress as they begin their cycle. But this optimism may well not reflect their overall level of psychological health, even from weeks before. Thus, using long-term physiological measures, such as hair cortisol, might well be the best way to truly assess the strength of the relationship. And that research does indeed show a robust significant relationship between distress and treatment outcome.

Another way to investigate the stress/IVF relationship is to look at it from an intervention perspective. If psychological interventions designed to decrease distress in IVF patients are associated with higher pregnancy rates, isn’t that another method to prove that, in fact, stress is associated with lower pregnancy rates from IVF?

The relationship between psychological interventions and pregnancy rates

There have been several meta-analyses published on the relationship between psychological interventions and pregnancy rates. One, published in 2015, included 39 studies on 2746 male and

female infertility patients (not inclusive only of IVF patients) [26]. The authors noted that “statistically significant and robust overall effects of psychosocial intervention were found for both clinical pregnancy ($p < 0.001$) and combined psychological outcomes ($p < 0.001$).” This same research group published another meta-analysis in 2021 on 15 studies with 2434 participants receiving IVF treatment [27]. Once again a positive association between psychological interventions and pregnancy rates was reported ($p = 0.033$). The authors noted that interventions which focused on skills acquisition, such as cognitive behavioural therapy (CBT) and/or mind/body interventions appeared to be the most efficacious.

However, two other meta-analyses don’t come to the same conclusions. A 2016 study included 20 randomized controlled trials on infertility patients, and although there were some studies which reported positive impacts of psychological interventions on distress and pregnancy rates, the authors concluded that there were methodological issues with every study [28]. In addition, the authors of a 2016 Cochrane review which included 39 studies on 4925 participants concluded that any impact of a psychological intervention is uncertain because of the numerous methodological issues in the field [29].

Is there another way to approach the stress/IVF outcome relationship?

It is well known that infertility can cause high levels of distress; most patients report some degree of sadness, frustration, isolation, irritability, and anxiety. However, because of the reasons just described, it might never be possible to truly know if distress can in fact lower pregnancy rates from IVF. What is clear though is that our patients are suffering—the number one reason why insured patients drop out of treatment prior to achieving a pregnancy is the emotional burden of treatment [30].

Treatment termination in insured patients is perplexing to many. Most clinics don’t track why or even how many of their patients don’t return. But the numbers of insured patients who drop out of treatment can approach 50% [31]. Even research which documented that a minor psychological intervention, a mailed packet of stress management and relaxation instructions—which cost \$12, could reduce dropout behaviour by 67%, and could significantly improve quality of life and reduce anxiety—was not incorporated into care [31].

An innovative approach to the stress/IVF outcome relationship question

Instead of continuing to research and subsequently “discuss” (i.e. argue about) whether or not stress negatively impacts IVF outcome, perhaps it is time to approach the question from a different direction. If one makes the assumption that infertility patients experience high levels of anxiety and depression, and this isn’t up for debate given the definitive research over the past ten or more years, and further makes the assumption that highly distressed patients are more difficult to care for, and far more likely to drop out of treatment, the direction of the inquiry should be addressed to a far more important direction—determine the most impactful and cost-effective way to decrease distress with our patient population. Here are ways to approach this challenge:

Encourage patients to acquire tools and strategies to make the process less emotionally taxing. Psychological interventions can indeed lead to significantly lower levels of distress [26, 27]. There are current efforts to make such interventions more accessible with mobile apps and online interventions. In a recent

randomized controlled study on the use of an online mind/body programme, the intervention patients experienced significant improvements on all assessments of distress and almost a four-fold increase in pregnancy [32].

Make efforts to minimize the emotional burden of infertility treatment through a variety of measures, including checking in on patients who have not returned to the clinic, understanding which patients might be at greatest risk for psychological distress, making attempts to include the partner in every way possible, examining the current treatment protocols and working to simplify them and/or incorporate effective preparation and education, having a mental health counsellor available to distressed patients, making stress management resources available, increasing patient sense of control through sharing decision-making, providing gentle counselling about lifestyle risk factors, and supporting and encouraging lifestyle changes in a non-judgmental and gentle fashion.

Learn empathic communication strategies. Infertility patients crave better communication from their infertility specialist. The most common complaint reported by infertility patients is that their physician lacked empathy [33]. Infertility physicians who learned how to communicate more empathetically were perceived by patients as providing better care and can also be perceived as spending more time with their patients [33].

The Covid-19 pandemic and infertility patients

For many infertility patients, there was a double impact of the pandemic; many clinics globally shut down in the spring of 2020 following the recommendations of ASRM and ESHRE, and infertility patients were cautious about the risks of exposure in the open clinics, as well as the increasingly alarming reports of the risks of contracting Covid-19 during pregnancy.

For patients who had their treatment cycles cancelled mid cycle or postponed, the psychological impact was high. In a study of 524 women and men who had their cycle cancelled or delayed, women reported significantly more distress than men and the scores were highest in women over 35 and those with a previous IVF failure [34].

In another study of 168 patients whose treatment was suspended in early April 2020, 72% wanted to resume treatment at the time of the study, none of the demographic characteristics correlated to distress, and the most distressed patients reported feeling the most helpless ($p < 0.01$) [35]. The patients who reported the least distress were those who had higher perceived social support and a greater sense of self-mastery ($p < 0.01$).

In another study of 627 patients whose treatment was stopped due to the pandemic, women reported higher levels of anxiety, depression, and distress than men [36]. The majority of patients wanted to resume treatment (65%) and those who had a relative impacted by Covid-19 reported significantly more distress than those with healthy relatives.

In a large study of 2202 infertility patients assessed in April 2020, with both retrospective and prospective data collection points, when asked to rate the most frequent stressor in their lives, participants noted infertility as the most frequent top stressor for all time points [37]. Coronavirus was cited as the third most common stressor for March 2020, but during the April 2020 surge, was similar to infertility. Only 6% of patients in April 2020 agreed that infertility treatment should not be offered during the pandemic.

In a further analysis of the Covid-19 study just cited [37], patients who were pregnant following ART treatment were sent

the same three questionnaires and were significantly more likely to cite the pandemic as their top stressor than infertility patients ($p < 0.001$) but were also significantly less likely to be practicing any stress-relieving activities [38].

The pandemic has also apparently drastically changed infertility patient interest in participating in telehealth visits. In a study of 1119 women undergoing infertility treatment who were surveyed both in April 2020 and 1 January 2021, 58% reported in the first survey that “in person” was their choice for their physician consult appointments, but in January 2021, the most common preference was “video telemedicine” (53.4%) ($P < 0.001$) [39].

Conclusions

Although it is clear that infertility is associated with high levels of emotional distress, whether or not stress can negatively impact infertility treatment is less certain. The investigation into that relationship is largely dependent on patient self-report of distress levels, which can be impacted by a variety of factors, including a desire to fake-good, the timing of the data collection, a sense of optimism at cycle start, the impact of lifestyle behaviours, and other fertility-related conditions such as PCO and endometriosis, which can have an independent impact on psychological health, regardless of fertility.

The recent research on physiological factors, especially hair cortisol levels, does present convincing evidence that stress may in fact have a negative impact on IVF outcome, but further research is needed.

Whether or not psychological interventions can have a positive impact on pregnancy rates in accordance with treatment is also somewhat controversial, although interventions which include specific coping strategies such as CBT or mind/body techniques, seem to be the most effective. There are multiple recent random controlled trials (RCTs) which do show a significant positive impact on pregnancy rates.

However, choosing to continue the debate as to whether or not psychological factors are associated with treatment failure doesn't seem to benefit anyone. Isn't now the time to focus time, energy, and resources on how best to minimize that distress? If every IVF patient could be offered the opportunity to learn strategies and skills to cope far more effectively with the demands of their infertility and subsequent treatment, they would be less distressed, far easier to care for, be less likely to drop out of treatment, and potentially more likely to get pregnant. No down side.

Thus, in conclusion, there does seem to be a role of stress in adversely affecting the treatment outcome of IVF patients, and interventions which focus on stress reduction may serve to not only decrease distress but also may increase pregnancy rates. The main goal in this field should be to stop investigating whether or not there is in fact a connection and instead put all resources into designing and implementing the most efficacious ways to lessen distress in this patient population.

References

1. Pasch LA, Holley SR, Bleil ME, Shehab D, Katz PP, Adler NE. Addressing the needs of fertility treatment patients and their partners: Are they informed of and do they receive mental health services? *Fertil Steril*. 2016;106:209–15.
2. Chen TH, Chang SP, Tsai CF, Juang KD. Prevalence of depressive and anxiety disorders in an assisted reproductive technique clinic. *Hum Reprod*. 2004;19:2313–8.

3. Berardis D, Mazza D, Marini M, Nibletto D, Serroni L, Pino N, Valchera MC, Ortolani A, Ciarrocchi C, Martinotti F, Giannantonio D. Psychopathology, emotional aspects and psychological counseling in infertility: A review. *Clin Ter.* 2014;165:163–9.
4. Smeenk JM, Verhaak CM, Eugster A, et al. The effect of anxiety and depression on the outcome of in-vitro fertilization. *Hum Reprod.* 2001;16(7):1420–3.
5. Eugster A, Vingerhoets AJJM, van Heck GL, et al. The effect of episodic anxiety on an in vitro fertilization and intracytoplasmic sperm injection treatment outcome: A pilot study. *J Psychosom Obstet Gynaecol.* 2004;25(1):57–65.
6. Ebbesen SMS, Zachariae R, Mehlsen MY, et al. Stressful life events are associated with a poor in-vitro fertilization (IVF) outcome: A prospective study. *Hum Reprod.* 2009;24(9):2173–82.
7. Kakatsaki D, Vaslamatzis G, Chatziandreou M, et al. Alexithymia is positively correlated with the outcome of in vitro fertilization (IVF) treatment. *Psychol Rep.* 2009;105(2):522–32.
8. Gourounti K, Anagnostopoulos F, Vaslamatzis G. The relation of psychological stress to pregnancy outcome among women undergoing in-vitro fertilization and intracytoplasmic sperm injection. *Women Health.* 2011;51(4):321–9.
9. Yuan A, Zhuangzhuang S, Li L, et al. Relationship between Psychological stress and reproductive outcome in women undergoing in vitro fertilization treatment: Psychological and neurohormonal assessment. *J Assist Reprod Genet.* 2013;30(1):35–41.
10. Haimovici F, Anderson JL, Bates GW, et al. Stress, anxiety, and depression of both partners in infertile couples are associated with cytokine levels and adverse IVF outcome. *Am J Reprod Immunol.* 2018;79(4):e12832.
11. Aimagambetova G, Issanov A, Terzic S, et al. The effect of psychological distress on IVF outcomes: Reality or speculation? *PLoS One.* 2020;15(12):e0242024.
12. Lintsen AME, Verhaak CM, Eijemans MJC, et al. Anxiety and depression have no influence on the cancellation and pregnancy rates of a first IVF or ICSI treatment. *Hum Reprod.* 2009;24(5):1092–8.
13. Pasch LA, Gregorich SE, Katz PK, et al. Psychological distress and in vitro fertilization outcome. *Fertil Steril.* 2012;98(2):459–64.
14. Zaig I, Azem F, Schreiber S, et al. Women's psychological profile and psychiatric diagnoses and the outcome of in vitro fertilization: Is there an association? *Arch Womens Ment Health.* 2012;15(5):353–9.
15. Cesta C, Johansson ALV, Hreinsson J, et al. A prospective investigation of perceived stress, infertility-related stress, and cortisol levels in women undergoing in vitro fertilization: Influence on embryo quality and clinical pregnancy rate. *Acta Obstet Gynecol Scand.* 2018;93(3):258–68.
16. Maroufizadeh S, Navid B, Omani-Samani R, et al. The effects of depression, anxiety and stress symptoms on the clinical pregnancy rate in women undergoing IVF treatment. *BMC Res Notes.* 2019;12(1):256.
17. Peng M, Wen M, Jiang T, et al. Stress, anxiety, and depression in infertile couples are not associated with a first IVF or ICSI treatment outcome. *BMC Pregnancy Childbirth.* 2021;21(1):725.
18. Csemiczky G, Landgren BM, Collins A. The influence of stress and state anxiety on the outcome of IVF-treatment: Psychological and endocrinological assessment of Swedish women entering IVF-treatment. *Acta Obstet Gynecol Scand.* 2000;79(2):113–8.
19. Kalaitzaki A, Mavrogianaki S, Makrigiannakis A. A prospective cross-sectional study of the protective and risk factors of successful in vitro fertilization outcome: Preliminary results in a Greek sample. *J Obstet Gynaecol.* 2020;40(3):382–7.
20. Matthiesen SMS, Frederiksen Y, Ingerslev HJ, et al. Stress, distress and outcome of assisted reproductive technology (ART): A meta-analysis. *Hum Reprod.* 2011;26(10):2763–6.
21. Purewal S, Chapman SCE, van den Akker OBA. Depression and state anxiety scores during assisted reproductive treatment are associated with outcome: A meta-analysis. *Reprod Biomed Online.* 2018;36:646–57.
22. Nicoloro-SantaBarbara J, Busso C, Moyer A, Lobel M. Just relax and you'll get pregnant? Meta-analysis examining women's emotional distress and the outcome of assisted reproductive technology. *Soc Sci Med.* 2018;213:54–62.
23. Massey AJ, Campbell BK, Raine-Fenning N, et al. Relationship between hair and salivary cortisol and pregnancy in women undergoing IVF. *Psychoneuroimmunology.* 2016;74:397–405.
24. Santa-Cruz DC, Caparros-Gonzalez RA, Romero-Gonzalez B, et al. Hair cortisol concentrations as a biomarker to predict a clinical pregnancy outcome after an IVF cycle: A pilot feasibility study. *Int J Environ Res Public Health.* 2020;17(9):3020.
25. Massey AJ, Campbell B, Raine-Fenning N, et al. The association of physiological cortisol and IVF treatment outcomes: A systematic review. *Reprod Med Biol.* 2014;13(4):161–76.
26. Frederiksen Y, Farver-Vestergaard I, Skovgard NG, Ingerslev HJ, Zachariae R. Efficacy of psychosocial interventions for psychological and pregnancy outcomes in infertile women and men: A systematic review and meta-analysis. *BMJ Open.* 2015;5(1):1–18.
27. Katyal N, Poulson CM, Knudsen UB, et al. The association between psychosocial interventions and fertility treatment outcome: A systematic review and meta-analysis. *Eur J Obstet Reprod Biol.* 2021;259:125–32.
28. Ying L, Wu LH, Loke AY. The effects of psychosocial interventions on the mental health, pregnancy rates, and marital function of infertile couples undergoing in vitro fertilization: A systematic review. *J Assist Reprod Genet.* 2016;33(6):689–701.
29. Verkuijlen J, Verhaak C, Nelen WLDM, et al. Psychological and educational interventions for subfertile men and women. *Cochrane Database Syst Rev.* 2016;3(3):CD011034
30. Lande Y, Seidman DS, Maman E, Baum M, Hourvitz A. Why do couples discontinue unlimited free IVF treatments? *Gynecol Endocrinol.* 2015;31:233–6.
31. Domar AD, Gross J, Rooney K, Boivin J. Exploratory randomized trial on the effect of a brief psychological intervention on emotions, quality of life, discontinuation, and pregnancy rates in in vitro fertilization patients. *Fertil Steril.* 2015;104:440–51.
32. Clifton J, Parent J, Seehaus M, et al. An internet-based mind/body intervention to mitigate distress in women experiencing infertility: A randomized pilot trial. *PLOS One.* 2020 Mar 18;15(3):e0229379.
33. Garcia D, Baustista O, Venereo L, et al. Training in empathic skills improves the patient-physician relationship during the first consultation in a fertility clinic. *Fertil Steril.* 2013;99:1413–8.
34. Barra F, La Rosa VL, Vitale SG, et al. Psychological status of infertile patients who had *in vitro* fertilization treatment interrupted or postponed due to COVID-19 pandemic: A cross-sectional study. *J Psychosom Obstet Gynaecol.* 2022;43(2):145–52.
35. Ben-Kimhy R, Youngster M, Medina-Artom TR, et al. Fertility patients under COVID-19: Attitudes, perceptions, and psychological reactions. *Hum Reprod.* 2020;35(12):2774–83.
36. Esposito V, Rania E, Lico D, et al. Influence of COVID-19 pandemic on the psychological status of couples. *Eur J Obstet Gynecol Reprod Biol.* 2020;253:148–53.
37. Vaughan D, Shah J, Penzias A, et al. Infertility remains a top stressor despite the COVID-19 pandemic. *Reprod Biomed Online.* 2020;41(3):425–7.
38. Domar A, Shah J, Gompers A, et al. The psychological impact of the COVID-19 pandemic on women pregnant following infertility treatment: A longitudinal study. *F S Rep.* 2022;3(1):71–8.
39. Vaughan DA, Yin SH, Shah JS, Gompers A, Hacker MR, Sakkas D, Domar AD, Toth TL. Telehealth for reproductive medicine: Pandemic and beyond. *J Assist Reprod Genet.* 2022;39(2):327–9.

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Introduction

Risk and safety management has become a science in its own right that today accompanies most modern enterprises. Medicine is no exception [1, 2]. Risk and safety management aims not at eradicating errors and mistakes—humans make mistakes and always will do—but at managing mistakes so that their most dreadful consequences can be foreseen and avoided [1]. This is accomplished by proper understanding, anticipation, and implementation of targeted measures—defences. Understanding risk and safety management ultimately aspires to developing a safety culture that accompanies all medical teams—in this case, assisted reproduction technology (ART). In this endeavour, one should largely count on education as a privileged vector for inoculating the desired safety culture deep into the daily activities of our working groups [3].

Safety management systems

Hazards and risks

Hazards are circumstances that constitute a potential source of danger. For example, in mountain climbing, the mountain itself is a hazard (Figure 75.1). Mountains being what they are, slipping while climbing can have far more dreadful consequences than if the same occurs in low lands. In this example, the mountain is the hazard that impacts on the consequences of other events, such as possibly slipping. This example illustrates the fact that hazard is a parameter that cannot be mitigated. Hazards are inherent to given processes and have to be dealt with.

Distinct from existing hazards, a risk is the possibility that something having unpleasant consequences happens. In our mountain climbing example, slipping is a risk. This risk—its practical consequences—is impacted by the prevailing hazard—the mountain. Indeed, slipping on top of a mountain can have consequences that are influenced by the nature of the hazard—the height of the mountain and the nature and proximity of its cliffs, among other factors. Mitigating the risk—reducing the chances that the risk materializes and/or reducing its consequences—will aim at altering the chance of slipping and avoiding what may ensue—a dramatic fall. However, as was said, hazards—the mountain in our example—have to be accounted for. Hazards impact on risks, but stand as facts that cannot be altered.

Defences are measures that aim at preventing risk from materializing and/or reducing the severity of their consequences [4]. In the case of mountain climbing, roping is an effective defence against the consequences—a possible fatal fall—that slipping may have (Figure 75.1). In the example given, we see that choosing the proper defence—here roping—implies an intimate understanding of both the risk and prevailing hazard, which modulates the seriousness of the risks [4]. Ultimately, defences ought to be judiciously chosen for not interfering too much with the task to be performed—here mountain climbing—and yet being as effective

as possible for avoiding the most serious consequences—here a fatal fall [4].

Hazards in ART

When performing surgery, such as an oocyte retrieval in ART, the inherent hazard is linked to the fact that a needle penetrates the patient's natural protections against bleeding and infection, the protective layers of the body [5]. By deliberately entering a needle into the pelvis for the purpose of retrieving oocytes, one confronts a hazard that is inherent to the measure taken. There are no other ways of performing oocyte retrievals, however, so entering a needle into the pelvic cavity cannot be avoided in ART. Just like the mountain is a hazard that needs to be taken as a fact, inserting needles into the pelvic cavity is a hazard inherent to the oocyte retrieval process itself [6]. One can only mitigate the risk of a catastrophic haemorrhage that might result from vascular damage by either preventing it happening or proactively managing its consequences if it does happen. We will conduct several risk analyses for the three categories of risk that exist in ART [5]. The objective is to show how proper understanding of risks helps with deploying the best defences for avoiding possible catastrophic consequences and thereby practicing safe medicine.

Know your risks in ART

Risks are dynamic processes generally evolving towards increasingly serious consequences [7]. Risks are commonly mapped on a severity versus likelihood diagram (Figure 75.2). Slipping on a mountain can have increasingly serious consequences depending on the proximity to the cliff and/or the ability to stop the slipping process early. Likewise, a haemorrhage is a dynamic process that will evolve from a minor self-contained event—the incident—to a catastrophic, possibly fatal accident. On the risk diagram, the consequences of the risk will move towards the less likely and more severe as we progress down and to the right [8]. Clinical management ought to maintain the course of risk complications within the green or possibly yellow parts of the diagram. In this analysis, one should emphasize the fact that adverse events such as post-oocyte retrieval haemorrhage cannot be avoided, no matter how careful one is. Hence, the protection against catastrophic outcome is not being careful in preventing haemorrhage occurring, but rather proper handling if it occurs. Patients, their spouses, and the whole team need to be trained to react accordingly, as here the ultimate safety—avoiding dreadful consequences—resides in managing haemorrhages, not preventing them.

The dynamic characteristics and the ability to detect the occurrence and progression—symptoms, laboratory findings, etc.—are specific for each risk. In the case of post-retrieval haemorrhage, one relies on symptoms such as pain, dizziness, and so on. In case of deep veno-thromboembolism (DVT), however, there are no announcing symptoms. Understanding the dynamics of each risk is therefore crucial for preventing catastrophic consequences. Post-oocyte retrieval haemorrhages can be followed



FIGURE 75.1 Hazards, risks, and defences in the mountain climbing example. Here the defence consists in roping.

clinically—symptoms exist—whereas avoiding DVT ought to revolve entirely on prevention in predefined high-risk patients. In the latter case, one solely relies on screening and initiating preventive treatment in identified high-risk women. The challenge therefore is to identify the patients who is at higher risk for DVT, knowing that if this identification fails, there are no symptoms to count on. Management of these two risks—haemorrhage and DVT—is therefore drastically different because the dynamics of these risks differ. Hence, as demonstrated for haemorrhage and DVT, each risk must be identified, understood, and its dynamic known. This is indispensable in order to adequately and effectively position protective measures—the defences—while minimizing possible interferences with the process itself.

Safety management systems in ART

A safety management system (SMS) is a formalized system of management of safety issues that has been rendered mandatory in aviation by its international supervising organization. Four sections are recognized in an SMS: (i) the definition of safety policies and objectives; (ii) safety and risk management, assessing all identified risks, knowing their characteristics, and

adopting adequate defences; (iii) safety assurance; and (iv) safety promotion. The safety level accomplished in the airline industry is such that SMS as it stands should inspire the development of safety systems that are adapted to the various segments of medicine. However, despite being inspired by the accomplishments achieved in aviation, this should be adapted to the specifics of the various segments of medicine, as copycat models simply will not work, considering the amount of differences between the two industries.

Risks in ART

Three categories of risks are recognized in ART: operational risks, functional risks, and personal risks (Figure 75.3).

Operational risks

Operational risks are linked to the procedure undertaken (i.e. oocyte retrieval). These risks are modulated by the hazard that consists of inserting a needle—for oocyte retrievals—into the pelvic cavity. We typically distinguish the risks of haemorrhage and of infection.

The risk of infection, post-ART tubo-ovarian abscess (TOA), is modulated by a new hazard—the now frequent presence of endometriomas, as surgery is not advocated anymore in these cases. Recent data have indeed pointed to the risk that ovarian surgery decreases ovarian reserve to the point of compromising responses to controlled ovarian stimulation (COS) and, in turn, ART outcomes. This has led to the now generalized practice of performing oocyte retrievals while endometriomas are in place, a hazard known to impact on the risk of TOA complications. Patients need to be made aware of this risk, including its possible late occurrence after ART [9]. Indeed, the primary defence against severe complications of TOA, such as ovariectomies, resides not in avoiding them, but rather in proper and prompt management by a team that is skilled in the art of managing such complications if they happen [9].

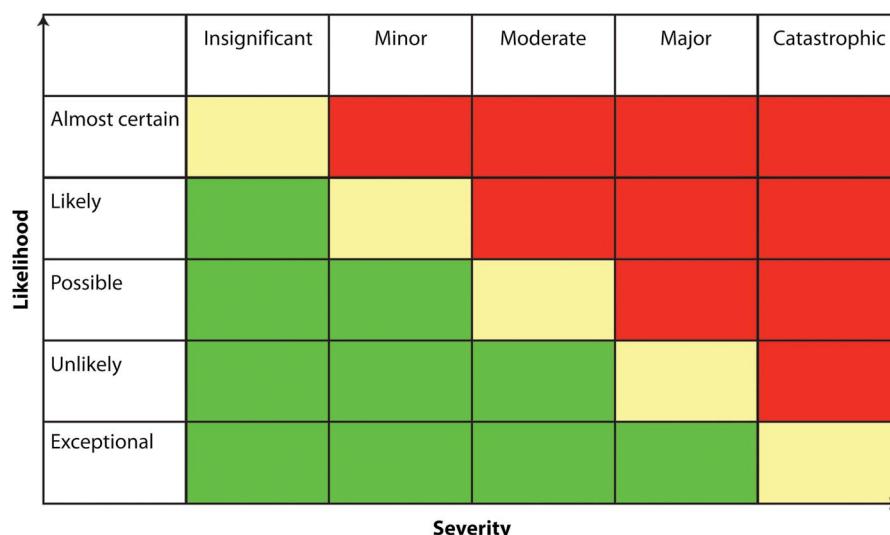


FIGURE 75.2 Know your risk. The consequences of any given risk result from a dynamic process that can be plotted on a likelihood versus severity diagram. In the case of post-oocyte retrieval haemorrhage, a slight increase in intrauterine bleeding (incident) may progress towards a dramatic, uncontrolled, possibly fatal haemorrhage, an unlikely but most severe event. The diagram serves to plot the parameters—symptoms and findings—that help recognize progression of the risk towards its lower right corner.

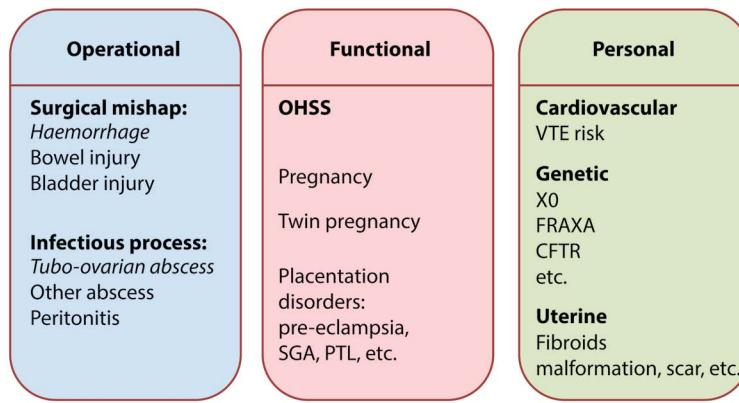


FIGURE 75.3 Three types of risk in assisted reproduction technology. Abbreviations: OHSS, ovarian hyperstimulation syndrome; PTL, preterm labour; SGA, small for gestational age; VTE, venous-thromboembolism.

Functional risks

Functional risks are possible adverse consequences of ART directly linked to the effects—hence, “functional”—of treatment used for inducing COS. First among these is the risk of ovarian hyperstimulation syndrome (OHSS) when the desired effect of treatment—multifollicular ovulation—is exceeded [10]. OHSS is a dreadful complication of ART that leads to a possibly fatal outcome. It is understood today that OHSS is not directly linked to multiple follicular development per se, but rather stems from an effect of human chorionic gonadotropin (hCG)—administered for triggering ovulation—on a large cohort of ovarian follicles [10]. Today, it is possible to nearly eradicate OHSS by refraining from using hCG in women who are at risk of OHSS, and rather reverting to gonadotropin-releasing hormone agonist for triggering ovulation, together with deferred embryo transfer.

Personal risks

Personal risks regroup possible adverse consequences encountered in ART that stem from various personal predispositions. These include three general categories of risks:

- *Venous-thromboembolism (VTE) risk.* Certain individuals are at increased risk of having intravascular clotting processes when exposed to hormonal imbalances such as those encountered in ART, notably elevated oestradiol levels. Women whose personal or family history is positive for past VTE episodes ought to be investigated with the objective of initiating protective measures—low-molecular-weight heparin treatment—during the course of ART and possibly pregnancy [11].
- *Genetic risk.* Certain genetic disorders associated with infertility can have dreadful consequence for the future child. An example of this is given by the possible *FRAXA* pre-mutation of the *FMR1* gene. In women, this disorder is known to cause primary ovarian insufficiency [12]. In the next generation, the *FRAXA* pre-mutation can transform into a full-blown mutation, which carries the risk of severe mental retardation in boys (fragile X) [12]. Premature ovarian weakness therefore warrants testing for the *FMR1* gene, calling for *ad hoc* pre-implantation or prenatal testing in positive findings. Several genetic risks have to be screened for in case of male factor infertility as well, notably the *CFTR* gene.

- *Uterine risk.* Constitutional (uterine malformation) or acquired conditions (large fibroids or past uterine surgery) can be associated with unwanted—possibly catastrophic (uterine rupture)—consequences during pregnancy. Proper counselling and precautions need to be implemented before undertaking ART.

Quality control and assurance

Simply put, enacting a quality control system—ISO 9001, Six Sigma, or others—in any industrial activity consists of reviewing the sum of processes undertaken, describing them in detailed documents, and subsequently ensuring that directives are followed. This can be summarized by the simple formula: “say what you do and do what you say.”

Practically, ISO 9001 is the quality control system most commonly chosen in ART. It implies creating a core management document, which contains all the necessary standard operation procedures (SOPs) that describe each and every step of what is done practically. Detailed SOPs describe, for example, the set of measures that are taken for selecting between different treatment steps and protocols based on the prevailing circumstances. For example, as ART outcomes decline with age, dealing with women whose ovarian response to COS is insufficient will differ depending on age. One will likely not pursue further ART treatments once it has been documented that a patient in the older age group had an insufficient response to COS. Conversely, *ad hoc* SOPs will describe that a different management should be applied when a seemingly similar event—poor response to COS—is encountered in a frankly younger patient.

Enacting a quality control system of ISO 9001-type controls for practice drifts over time, which could ultimately alter ART outcomes. If changes need to be enacted in ART management, new SOPs are prepared, distributed internally, and finally enacted. Later, outcomes—pregnancy rates and other relevant outcome parameters—can be assessed in order to determine the possible impact that the enacted change may have had. In case of a negative impact, it is easy to notice it and, if need be, to revert to the prior, possibly more effective process. By the nature of ART results—they hover between 0% and 100%, generally in the middle—ART is prone to fluctuations in outcomes, making it difficult to determine whether such changes in results are due to chance or a change having occurred in a given process. By its thorough

documentation, a quality control system of the ISO 9001 type allows us to rapidly account for possible practice changes. In many countries, including France, supervising bodies mandate that a quality control system is enacted for certifying ART programmes [13]. Most often, ART programmes have chosen ISO 9001 as their quality control system.

Lessons from aviation

Checklists

Everyone knows—and it is an emblematic figure of aviation—that pilots run checklists before performing crucial steps of their flights. This has been an unavoidable approach taken in order to ensure that no crucial steps and/or actions are overlooked at a time when the workload may be significant in the cockpit. Historically, it is the introduction of more complex airplanes—the Boeing B-17, to be specific—that led to the generalized introduction of formal checklists in aviation.

The soundness and efficacy of checklists as a safety measure have been widely recognized and, in recent years, exported to other industries, including medicine [14]. The mounting awareness and arising concerns about medical errors and their sometimes dreadful consequences have sparked efforts for introducing *ad hoc* checklists in the highest-risk segments of the medical environment, notably including operating rooms (ORs), intensive care units (ICUs), and delivery rooms (DRs). Insurance carriers and hospital administration have deployed remarkable efforts for introducing and enforcing the use of checklists in medical institutions, notably in ORs, ICUs, and DRs.

Checklists introduced at long last in medicine—the Boeing B-17 is a World War II-era bomber—have curbed the unacceptable series of mega-mistakes (notably, the infamous “triple W”—the irrecoverable wrong patient, wrong organ, and wrong side errors). Once these achievements are accomplished, however—indispensable as they are—checklists do little for reducing the larger part of medical errors, which occur in the doctor’s office. These revolve around making the wrong decision for undertaking a non-ideal treatment at a non-optimal time. Hence, once checklists are introduced—and they certainly need to be—it is important to go beyond that and address the root causes of medical errors that originate in the doctor’s office. Education, as discussed later, is one effective vector for bringing about a culture of safety in the doctor’s office.

From airmanship to medicalship

Airmanship is a word inspired from the seminal concept of seamanship that has long existed and inspired sailors. Like seamanship—the art of mastering navigation while taking all factors into account—airmanship describes the skill of mastering all that matters for safe and efficient flights.

A similar concept has been long awaited in medicine. “Medicalship” is the word coined for similarly defining the art of managing medicine as a global—series of strings, rather than “in slices”—juxtaposition of independent steps simply added one after the other. Procedures have implications that carry far beyond the limits of the procedure itself. For example, what are the therapeutic options for a woman suffering from infertility and endometriosis? Will this woman undergo surgery for removing her endometriotic lesions, or rather revert to ART? In which order will this be done? Knowing that surgery favours natural conception but not ART outcomes, one will enquire before offering surgery about her ovarian reserve and the spouse’s sperm. Does she have time for attempting

to conceive naturally after surgery if it is performed? Likewise, is the sperm quality compatible with natural conception? In the risk–benefit equation pertaining to surgery for endometriosis, the chance of conceiving naturally after surgery is the benefit that compensates for the cost and risk of surgery. If the chance of conceiving naturally cannot be met because there is no time for waiting for natural conception—perhaps due to impaired ovarian reserve—or the sperm is suboptimal, the risk of surgery is not balanced out. In these cases, surgery should not be opted for. This example illustrates how medical measures intricate themselves into one another and should be looked at as series of linkages—medicalship—rather than the isolated steps of “by-slices” medicine.

It is an active part of safety management in medicine to ensure that procedures are proposed in a medicalship-inspired philosophy and spirit. Medical procedures—diagnostic measures, treatment processes, and surgical procedures—need to be assessed dynamically as strings of mutually dependent procedures, rather than taken in isolation.

By-procedure operation versus resilience

Airlines have championed the concept of “by-procedure” operation. If weather conditions at the destination are below a minimum, approaches to landing are simply not flown and flights are diverted to alternative airports. Most often, little is left to interpretation, and pilots simply follow procedures. Moreover, when an approach to landing at a destination cannot be flown due to adverse conditions and the alternative airport is of no commercial interest for passengers, airlines may set internal procedures and simply cancel the flight. In this case, internal procedures (cancelling the flight) complement or supersede regulatory procedures (flying to an alternative airport).

The remarkable safety levels of airline operations—the safest mode of transportation—is, to a great part, dependent upon the by-procedure mode of operation that has ruled the airline industry. One requirement for relying on by-procedure operation, however, is the repetitive nature of these operations. This typically applies to airline flights. Very little is left to the unknown. However, this is not necessarily the case for all procedures in medicine, nor is it the case for certain non-airline aviation operations.

Non-repetitive tasks simply cannot rely on by-procedure operation alone. This notably includes certain air and medical operations [15]. For example, search and rescue air operations in mountainous terrain or high seas are too varying in nature and by essence not repetitive enough to be conducted on a by-procedure basis. Different from airline operations, search and rescue sorties engaged for salvaging endangered human life will have to count on the crew’s resilience as much as its adherence to procedures. Such operations generally take place in bad weather, because this is precisely when accidents occur. Search and rescue missions rely at times on the crew’s ability to improvise based on past experience and immediate analysis of the unique circumstances prevailing in each mission—a skill that is identified as resilience. Often, the circumstances prevailing in search and rescue missions are such that airline pilots would simply call off the flight. But in search and rescue missions, this might equate to death for the endangered mountaineers or seamen in distress. While search and rescue missions are not always possible, crews will nonetheless strive to achieve their utmost in order to deliver the impossible, always pushing the limits further. Predictably, search and rescue operations do not have the safety records of airlines, but do remarkably well in view of the circumstances, a fact that stands to inspire medicine as a whole.

Not all helicopter operations rely primarily on resilience like the extremes encountered in search and rescue missions, as already discussed. Supply to offshore drilling platforms, for example, is conducted with near-airline repetition and essentially on a by-procedure basis. If the weather is bad, supply will wait until the next day. Logically, helicopter operations to offshore platforms are accomplished with near-airline safety records. We see therefore that helicopter operations as a whole encompass a span of activities ranging from the extreme in search and rescue missions that call for unrestrained resilience to near-airline, by-procedure operation, in the case of supplies flown to offshore oil platforms. In that sense, helicopter operations—less known to the lay public than airlines—are better models for medicine. Indeed, medicine also includes a comparable diversity that ranges from extreme missions—surgery for cancer “all over”—to routine, by-procedure operations. In this spectrum of diverse medical operations, ART occupies the position of airline-like, by-procedure operations and should therefore achieve optimal safety records. While resilience can become handy in certain difficult, unpredictable circumstances, it should be sparsely and judiciously used in ART, which is in essence a by-procedure activity. We can see from the preceding discussion how ART should strike out as a reliable and efficient by-procedure operation that is capable of achieving near-airline safety records.

Differences between medicine and aviation

In the safety realm of aviation—SMS and quality control—passengers do not actively partake in the process. In aviation, passengers might as well be sandbags, as solely their mass is taken into account when conducting weight and balance calculations. This is not the case for patients, who cannot be ignored, as they clearly participate and can influence the whole safety process.

In aviation, only the crew is taken into account when assessing workload patterns encountered during the successive segments of flights, ensuring that a ceiling—excessive workload—is not exceeded. Typically, it is before initiating approaches to landing that the workload is at its highest for the crew, leading to the risk of exceeding an acceptable and safe ceiling. Awareness of this process allows the crew to take specific measures to prevent reaching this ceiling, such as through anticipation.

In ART operations, one can easily understand that too many oocyte retrievals falling on a given day may lead to an excessive workload for certain team members (clinicians, biologists, etc.) (Figure 75.4). The approach that needs to be undertaken in order to prevent this from happening will possibly include cycle synchronization with timely use of oral contraceptive (OC) or other measures in order to even out the number of ART cases conducted each week and to set it to a level that is acceptable for the whole group.

In ART, however, it is not just the medical team who can be put under an excessive workload—patients may be as well. If patients are overwhelmed (e.g. by too much information given at the same time) mistakes will occur (i.e. treatment errors). Clearly, patient mistakes can impact on the overall safety of the whole ART process. Patients are prone to encounter an excessive workload at times in the ART process that is different from the medical team. For example, patients could not care less about the number of retrievals performed on a given day. They only have one on that day—their own—and that is all that counts for them. Days before the retrieval, however, patients are given slews of information and lists of safety-inspired recommendations that may be excessive if not adequately planned. For example, before retrievals take place, you want to be sure that the patient’s spouse will take his wife home after the retrieval and stay with her for the whole first

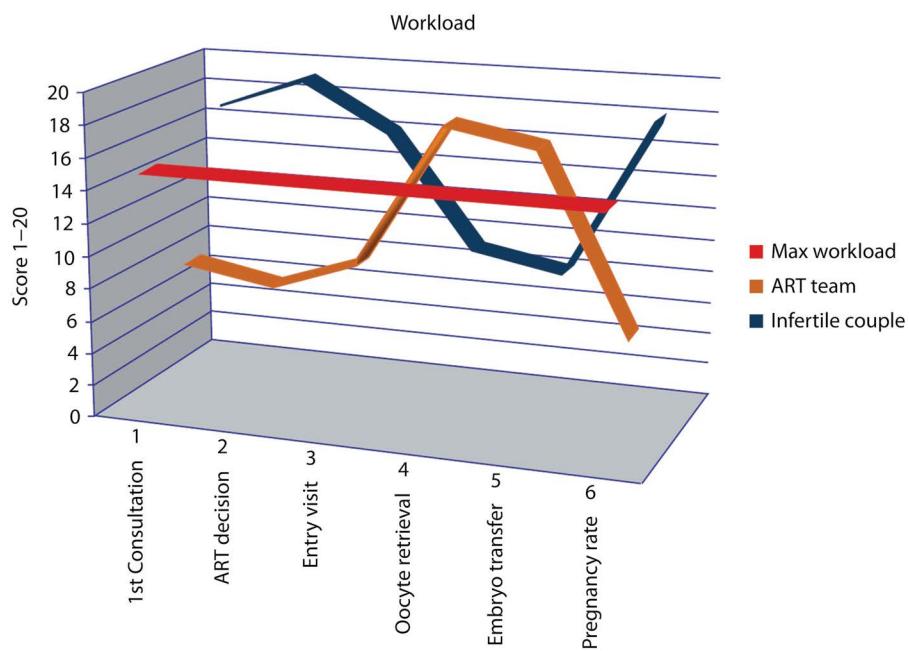


FIGURE 75.4 Workload pattern throughout the ART process. Workload increases at certain times in the ART process, and these increases are different for the various members of the medical teams. Cycle synchronization—using OC or other means—can help with avoiding reaching an excessive workload for team members (i.e. too many retrievals on a given day). Planning information that is given to patients can help with preventing them reaching an excessive workload and becoming overwhelmed by too much information being given at a specific time. Abbreviation: ART, assisted reproduction technology.

night. Recognizing the importance of this safety measure highlights how and particularly when patients have to be made aware of it. The best time for reminding patients of this measure is on the day of ovulation triggering, early enough for action to be taken and not too long before the retrieval day so as to incur the risk of the recommendation being forgotten. Awareness of the patient workload (Figure 75.4) is therefore a crucial safety step that is as important as the workload of the medical team. In the defence deployed against haemorrhage risks, the spouse is a key element in the whole safety link. Misinformation or information provided at an erroneous time deprives the patient of a key safety feature if, ultimately, the spouse is not with the patient during that first night. Safety of the whole process indeed includes the presence of the spouse for intervening—returning to the hospital—if need be. We see therefore that proper safety operation of outpatient procedures such as ART implies mastering the workload pattern—What? How much/many? When?—of both the medical team and patients. Each may encounter their limit with an excess workload—the safety ceiling. However, this will likely happen at distinct times in the ART process for patients and the medical team. Contrary to what prevails in aviation—passengers might as well be sandbags—patients need to be included as active partners in the workload analysis of an ART operation and therefore in the whole of safety management.

Education conceived as “safety inside”

Safety and, in particular, a safety culture cannot be force-fed to people who have long been managing their work operations individually with limited concerns for outside inputs into safety management. The perfect vector for inoculating a safety culture in medical operation is education [3], which can dispense new knowledge items laced with related pertinent safety issues. This is what we identify as education conceived as “safety inside,” by analogy to a certain microprocessor found “inside” computers of all kinds and makes.

Conclusion

Safety management is a science that has taken medicine by storm under the impetus of insurance companies and hospital administration. ART, a highly repetitive by-procedure operation, is no exception. The nature of ART as generally conducted in healthy individuals should be an example of ultimate safety achievement.

References

1. de Ziegler D, Gambone JC, Meldrum DR, et al. Risk and safety management in infertility and assisted reproductive technology (ART): From the doctor's office to the ART procedure. *Fertil Steril.* 2013;100:1509–17.
2. Meldrum DR, de Ziegler D. Introduction: Risk and safety management in infertility and assisted reproductive technology. *Fertil Steril.* 2013;100:1497–8.
3. de Ziegler D, de Ziegler N, Sean S, et al. Training in reproductive endocrinology and infertility and assisted reproductive technologies: Options and worldwide needs. *Fertil Steril.* 2015;104:16–23.
4. Boothman RC, Imhoff SJ, Campbell DA Jr. Nurturing a culture of patient safety and achieving lower malpractice risk through disclosure: Lessons learned and future directions. *Front Health Serv Manage.* 2012;28:13–28.
5. Schenker JG, Ezra Y. Complications of assisted reproductive techniques. *Fertil Steril.* 1994;61:411–22.
6. Nardelli AA, Stafinski T, Motan T, et al. Assisted reproductive technologies (ARTs): Evaluation of evidence to support public policy development. *Reprod Health.* 2014;11:76.
7. Guo J, Meng F, Ma N, et al. Meta-analysis of safety of the coadministration of statin with fenofibrate in patients with combined hyperlipidemia. *Am J Cardiol.* 2012;110:1296–301.
8. Schulman S, Spencer FA. Antithrombotic drugs in coronary artery disease: Risk benefit ratio and bleeding. *J Thromb Haemost.* 2010;8:641–50.
9. Villette C, Bourret A, Santulli P, et al. Risks of tubo-ovarian abscess in cases of endometrioma and assisted reproductive technologies are both under- and overreported. *Fertil Steril.* 2016;106:410–5.
10. Messini CI, Daponte A, Anifandis G, et al. Standards of care in infertility in Europe. *Eur J Obstet Gynecol Reprod Biol.* 2016;207:205–10.
11. Nelson SM. Venous thrombosis during assisted reproduction: Novel risk reduction strategies. *Thromb Res.* 2013;131(Suppl 1):S1–3.
12. Streuli I, Fraisse T, Ibecheole V, et al. Intermediate and premutation FMRI alleles in women with occult primary ovarian insufficiency. *Fertil Steril.* 2009;92:464–70.
13. Harper J, Geraedts J, Borry P, et al. Current issues in medically assisted reproduction and genetics in Europe: Research, clinical practice, ethics, legal issues and policy. *Hum Reprod.* 2014;29:1603–9.
14. Denham CR, Sullenberger CB 3rd, Quaid DW, et al. An NTSB for health care: Learning from innovation: Debate and innovate or capitulate. *J Patient Saf.* 2012;8:3–14.
15. Skinner H. Bad medicine: Resilience. *Br J Gen Pract.* 2017;67:13.



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 ICUs, *see* Intensive care units
 IFFS, *see* International Federation of Fertility Societies
 IGF, *see* Insulin like growth factor
 IGF-I, *see* Insulin-like growth factor I
 ILs, *see* Interleukins
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IoT, *see* Internet of things

IPD, *see* Individual patient data

IPD-MA, *see* Individual patient data meta-analysis

ISMAAR, *see* International Society for Mild Approaches in Assisted Reproduction

ISO, *see* International Organization for Standardization

IUA, *see* Intrauterine adhesions

IUI, *see* Intrauterine insemination
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