

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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This volume is available on its own (ISBN 9781032214801) or as part of the two-volume set (ISBN 9781032245348).



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CRC Press

Taylor & Francis Group

Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

Sixth edition published 2024

by CRC Press

6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487-2742

and by CRC Press

4 Park Square, Milton Park, Abingdon, Oxon, OX14 4RN

CRC Press is an imprint of Taylor & Francis Group, LLC

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ISBN: 9781032214764 (Vol. 1 hardback)

ISBN: 9781032214788 (Vol. 1 paperback)

ISBN: 9781003268598 (Vol. 1 eBook)

ISBN: 9781032214801 (Vol. 2 hardback)

ISBN: 9781032214856 (Vol. 2 paperback)

ISBN: 9781003268611 (Vol. 2 eBook)

ISBN: 9781032761695 (Vol 1. Indian edition)

ISBN: 9781032761701 (Vol 2. Indian edition)

ISBN: 9781032245348 (Two-volume set/Hardback)

ISBN: 9781032558578 (Two-volume set/Paperback)

ISBN: 9781032752877 (Indian edition, two-volume set/Hardback)

DOI: [10.1201/9781003268611](https://doi.org/10.1201/9781003268611)

Typeset in Warnock Pro
by KnowledgeWorks Global Ltd.

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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,
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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]:

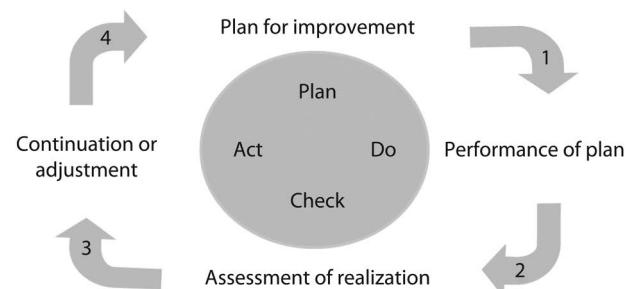
1. Revised Guidelines for Human Embryology and Andrology Laboratories, the American Fertility Society, 2008
2. Revised Guidelines for Good Practice in IVF Laboratories, the European Society of Human Reproduction and Embryology (ESHRE), 2008
3. Reproductive Laboratory Accreditation Standards, College 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.

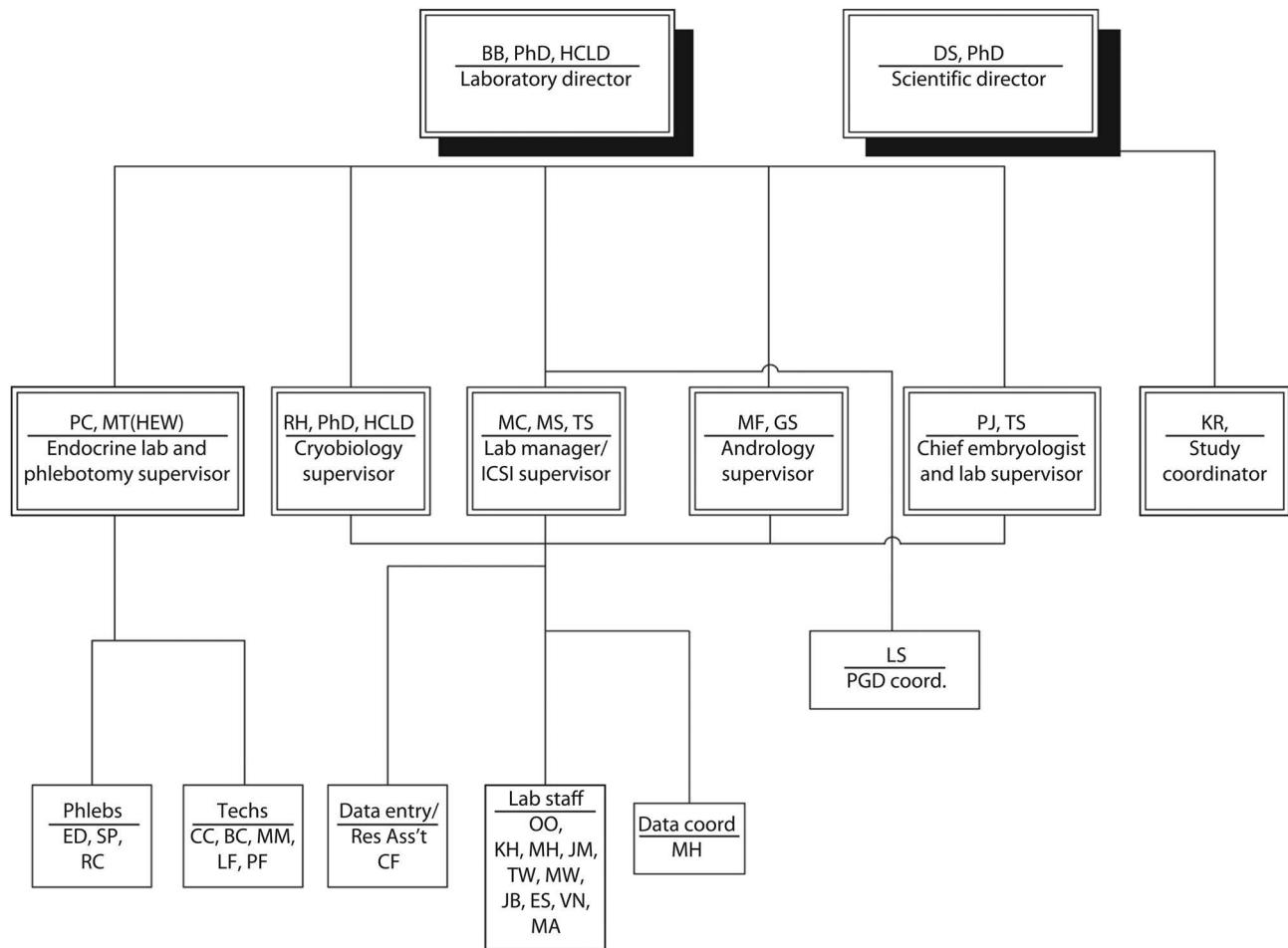


FIGURE 32.2 Example of an organizational chart.

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

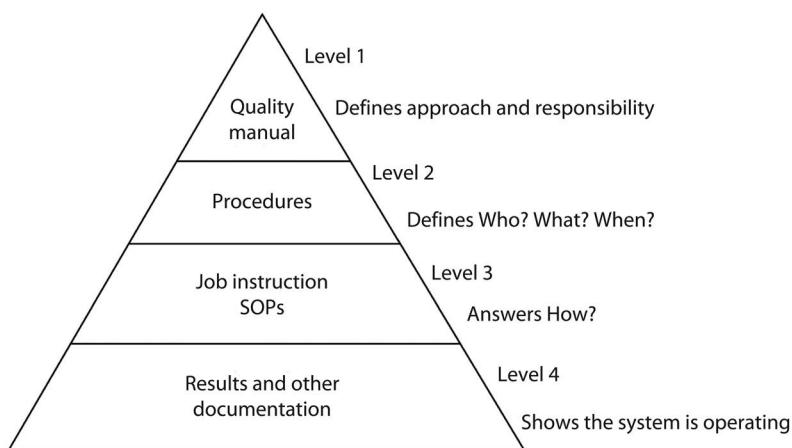


FIGURE 32.3 Levels of documentation. Abbreviation: SOP, standard operating procedure.

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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

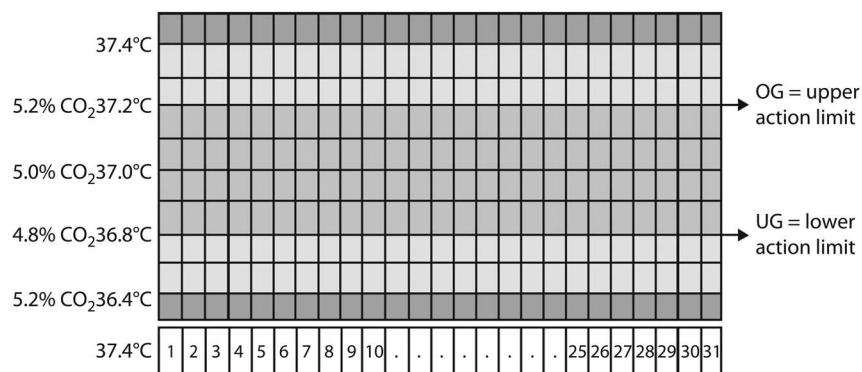


FIGURE 32.5 Monitoring temperature and CO₂ levels in an incubator.

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

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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.

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34

LIFESTYLE, PERICONCEPTION, AND FERTILITY

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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

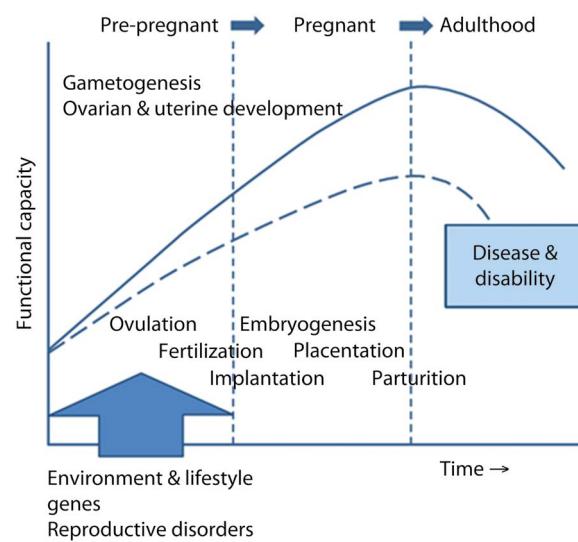


FIGURE 34.1 Adaptation to adverse influences in early life causes loss of functional capacity after birth.

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].

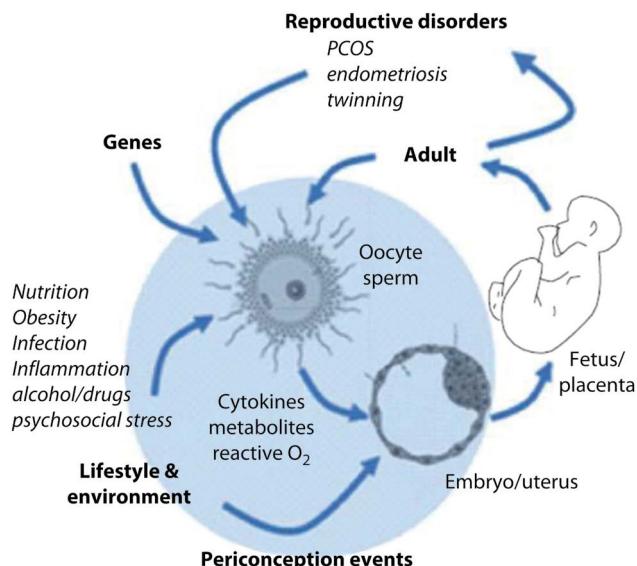


FIGURE 34.2 Periconception events are influenced by genes, a range of lifestyle/environmental factors, and maternal factors including reproductive disorders to impact fetal development and adult outcome.

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.

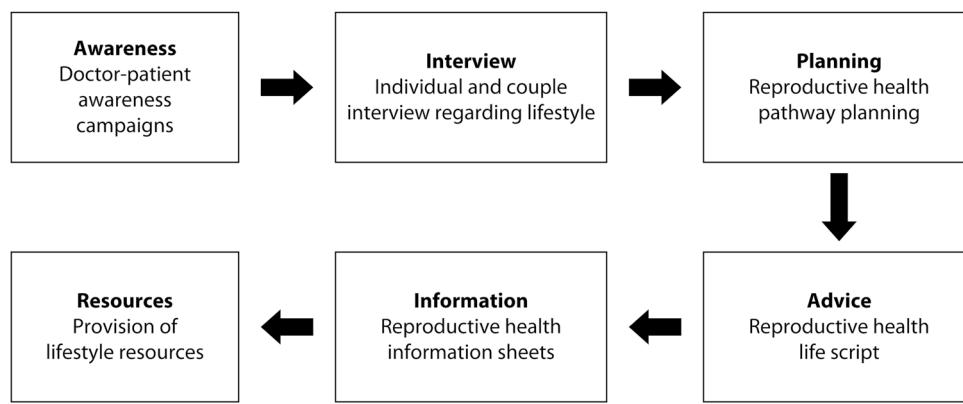


FIGURE 34.3 An approach to assessing and managing lifestyle in a clinical setting.

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.

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35

HUMAN REPRODUCTION ACROSS THE LIFE COURSE AND THE TOTAL ENVIRONMENT

Leah Martin, Yu Zhang, Irene Souter, and Carmen Messerlian

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

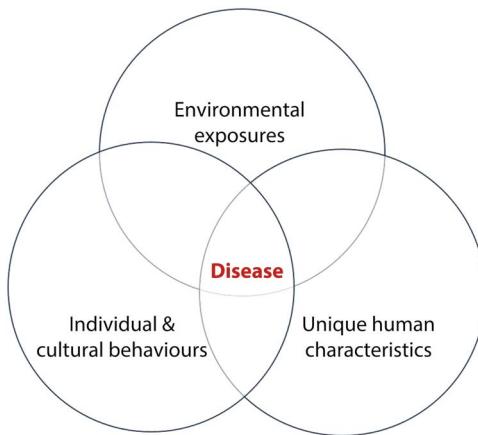


FIGURE 35.1 The exposome: Major components contributing to disease.

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

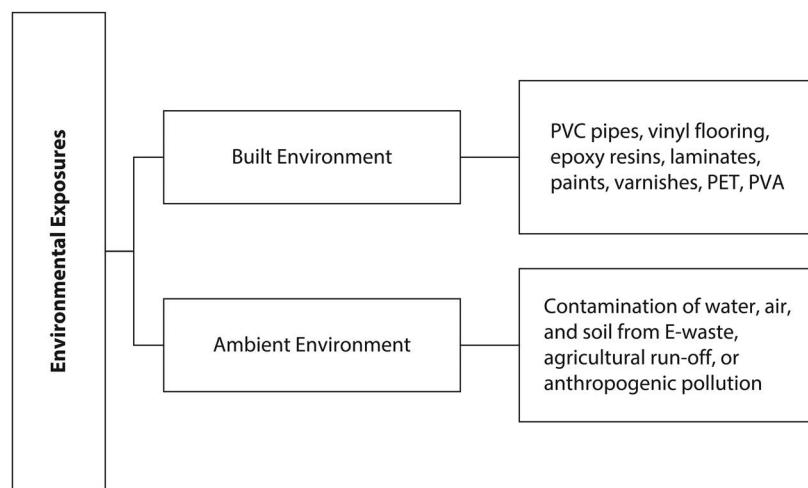


FIGURE 35.2 Main routes of exposure to EDCs in the natural-built environment.

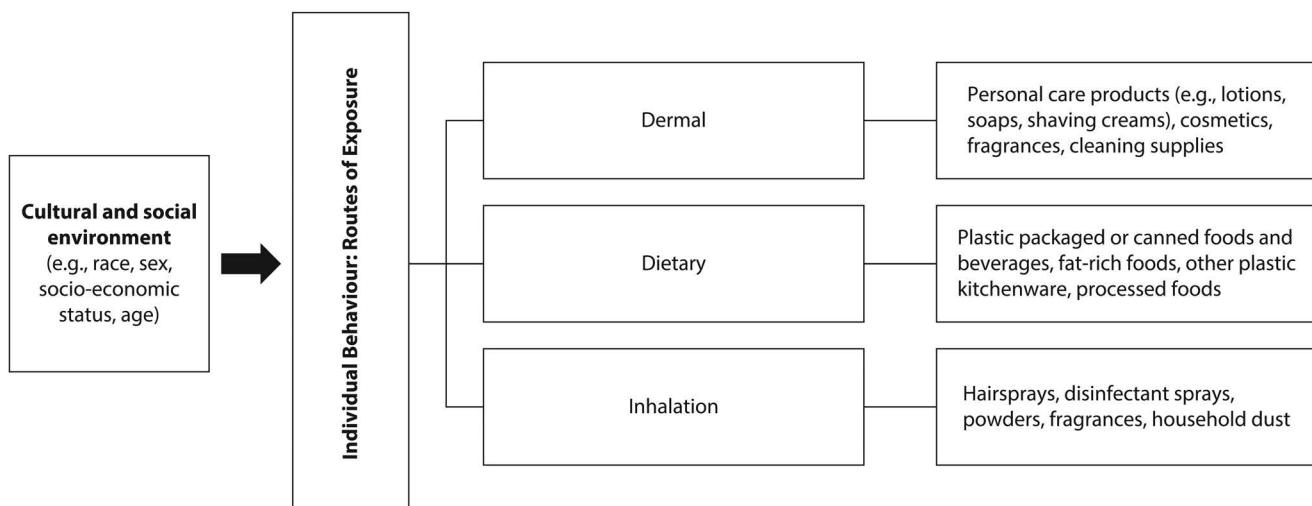


FIGURE 35.3 Possible primary routes of exposure related to cultural and social environments.

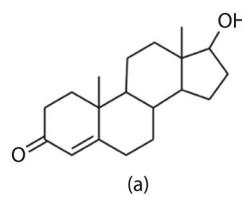
fats and oils, and can be absorbed and stored in fish, meat, dairy, breast milk, and other fat-rich foods. Fast foods also tend to have added flavours, hydrogenated oils, dyes, or hydrolyzed proteins that increase EDC content [6]. When consumed, these toxicants are absorbed by the digestive tract and 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].

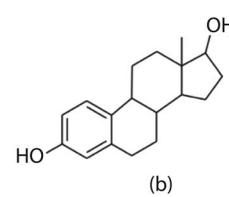
Another source of everyday EDC exposure often found within the natural-built environment includes electronic devices, such as cell phones and laptops, that emit radio frequency electromagnetic radiation (RF-EMR) and often contain EDCs to reduce flammability. While associations between RF-EMR exposure 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

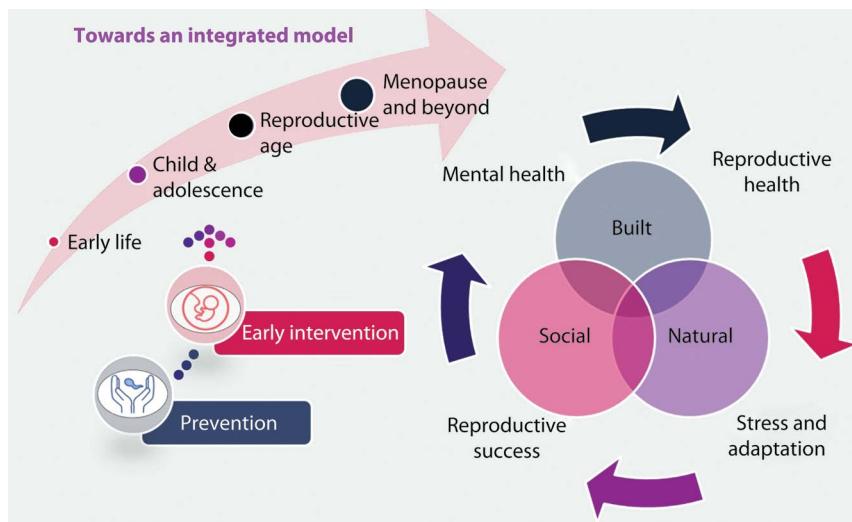
fine-tuning preventive medicine strategies, and improving reproductive health outcomes. During sensitive time periods of development certain environmental exposures can pose severe and often irreversible effects on systems, organs, tissues, and cells—ultimately impairing individual reproductive health, 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

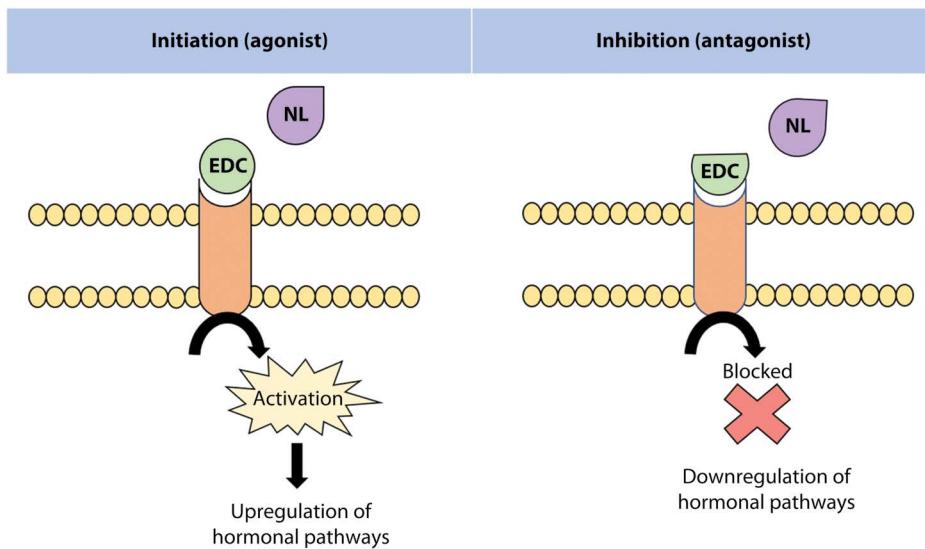


FIGURE 35.6 Examples of activation and inhibition of natural ligand (NL) by EDCs.

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

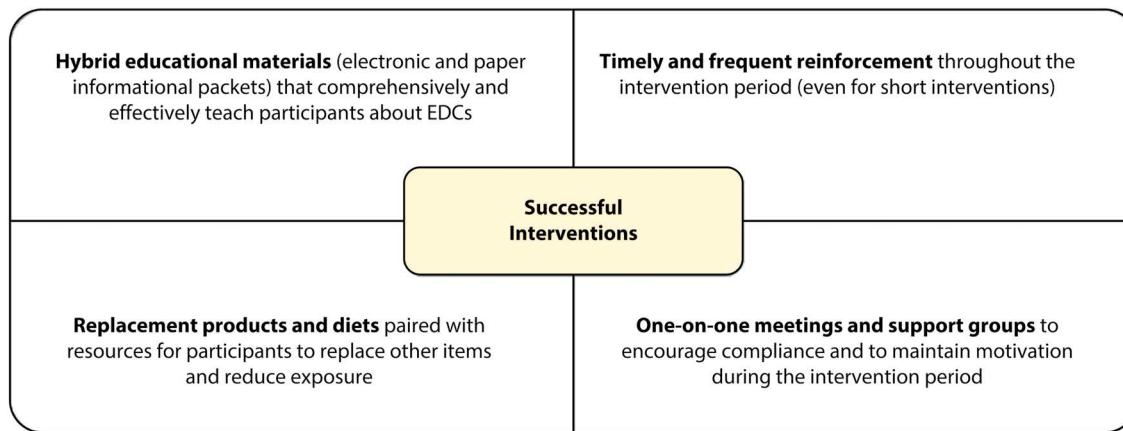


FIGURE 35.7 Successful strategies among current interventions to apply to future interventions.

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.

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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.

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INITIAL INVESTIGATION OF THE INFERTILE COUPLE

Ranit Hizkiyah, William Buckett, and Togas Tulandi

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.

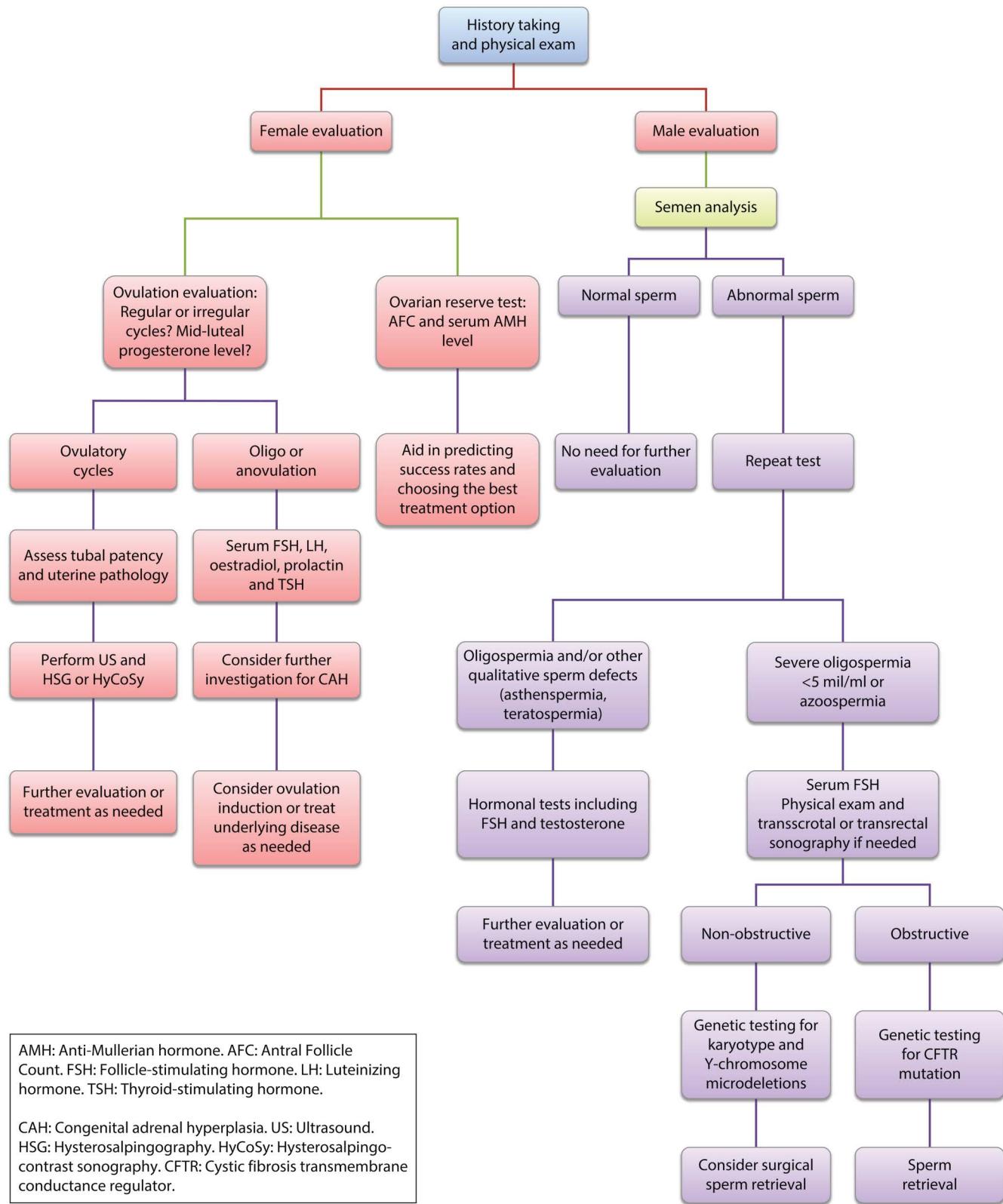
In case of agenesis of the vas deferens related to CFTR mutation, a history of non-severe pulmonary diseases or asthma may or may not be present. Concerning the genitalia, fibrous cord-like vas may be palpable, only the seminal vesicles and proximal vas may be missing, or asymmetry may be apparent [49].

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.

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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

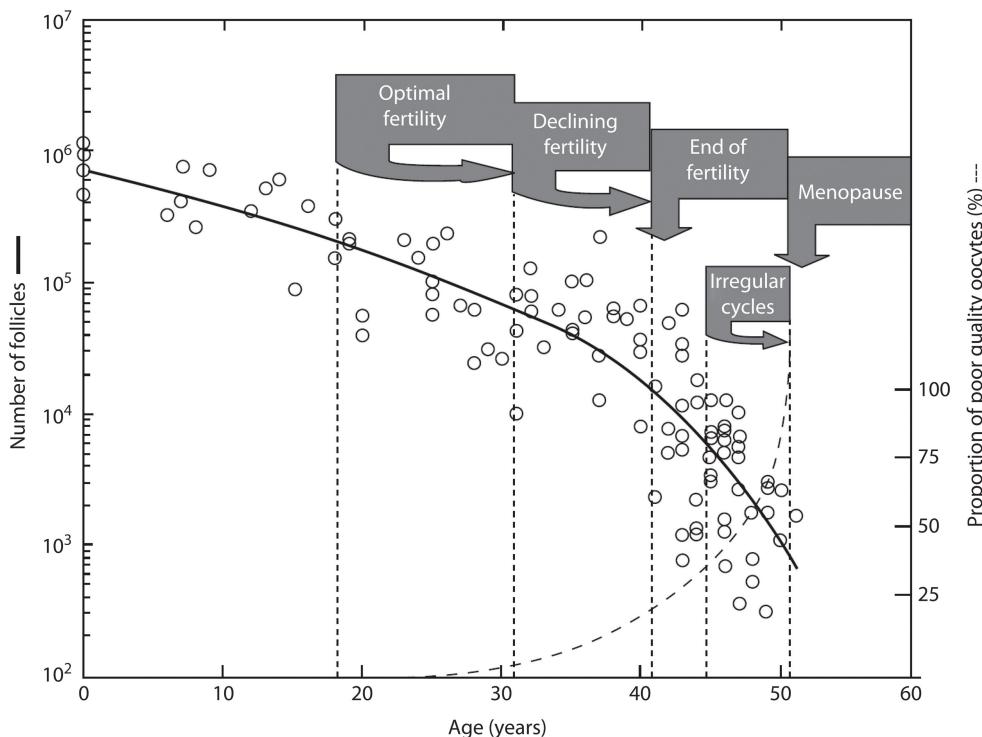


FIGURE 38.1 Quantitative (solid line) and qualitative (dotted line) declines of the ovarian follicle pool, which is assumed to dictate the onset of important reproductive events. (Adapted from [9].)

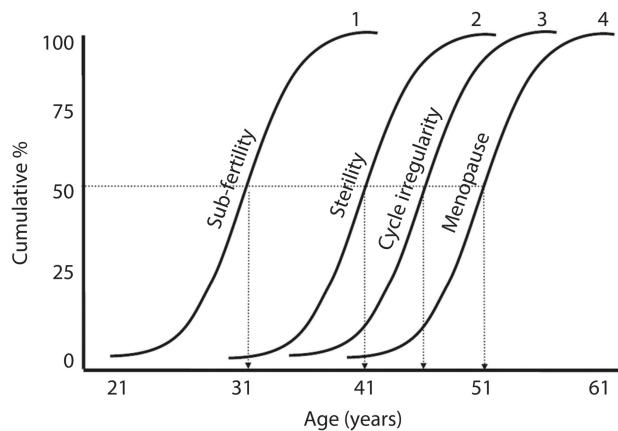


FIGURE 38.2 Variations in age at the occurrence of specific stages of ovarian aging. (For explanation of the background of data, see [2], with permission.)

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.

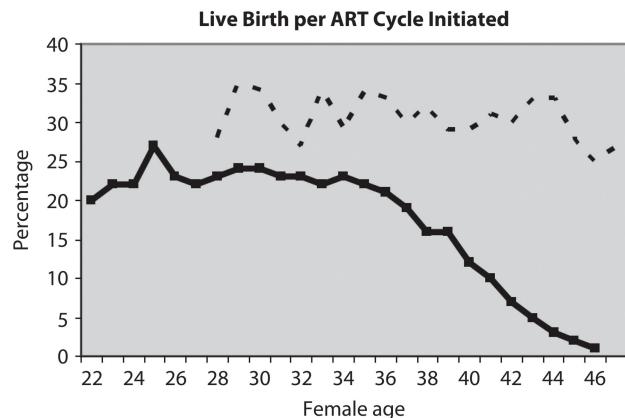


FIGURE 38.3 Effect upon average singleton live birth rates of female age, showing a steady decrease after the age of 34 years. The dotted line represents the average singleton live birth rate after oocyte donation as a function of the recipient age. It underlines the potential of oocyte donation in the 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

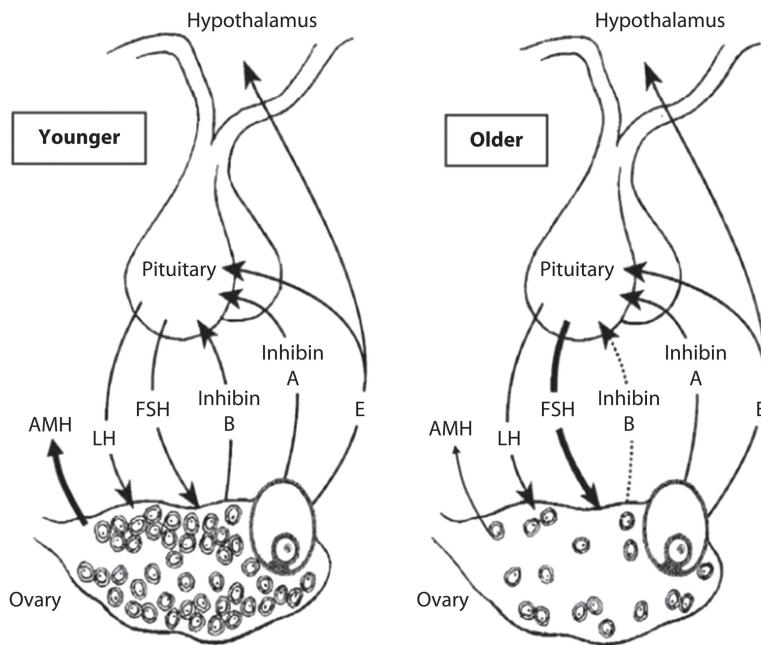


FIGURE 38.4 Illustration of the changes in follicle reserve with increasing female age and the effects of these quantitative changes upon several endocrine factors. Abbreviations: AMH, anti-Mullerian hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone. (Adapted from [21].)

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].

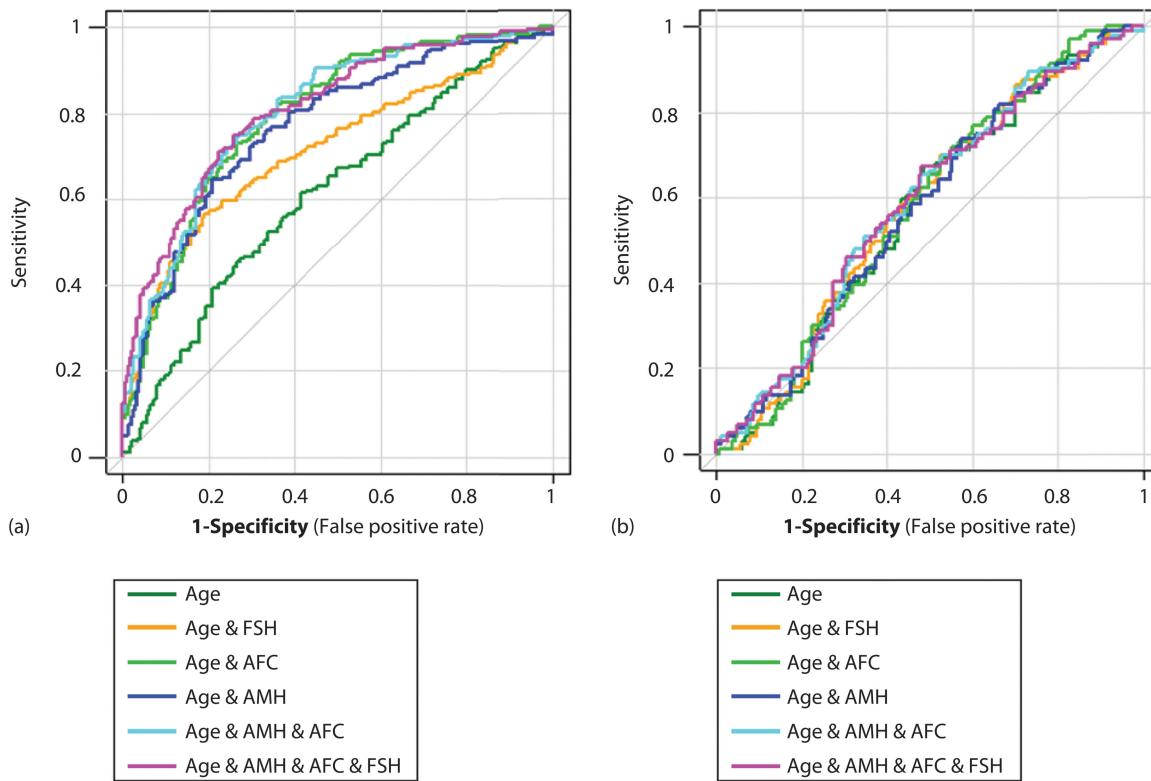


FIGURE 38.5 Receiver–operator characteristic (ROC) curves of age and ovarian reserve tests (ORTs) in the prediction of poor response and ongoing pregnancy. (a) Poor-response prediction based on age and ORT. The ROC curves of age or age combined with one or more ORT are depicted. The ROC curves for “Age + 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.

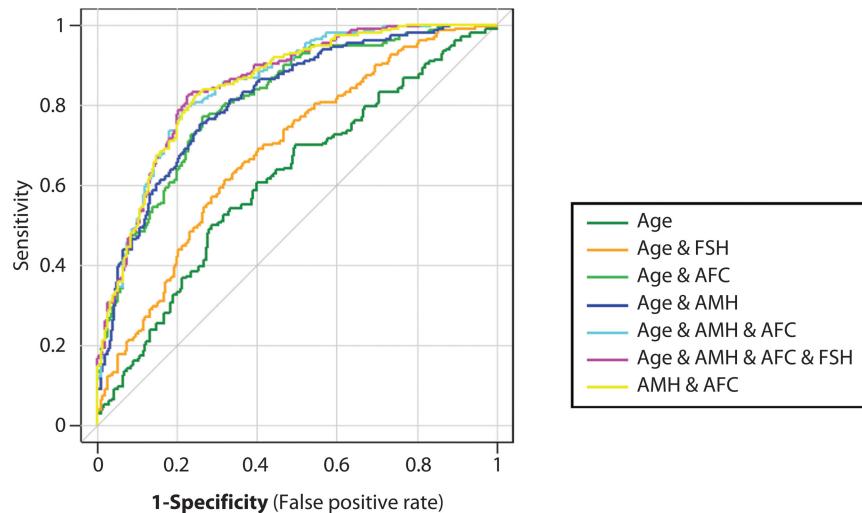


FIGURE 38.6 Receiver-operator characteristic (ROC) curves of age and ovarian reserve tests in the prediction of an excessive response. The ROC curves of age and age combined with one or more ovarian reserve test are depicted. The ROC curves for “Age + AMH,” “Age + AFC,” “Age + AMH + AFC,” and “Age + 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.

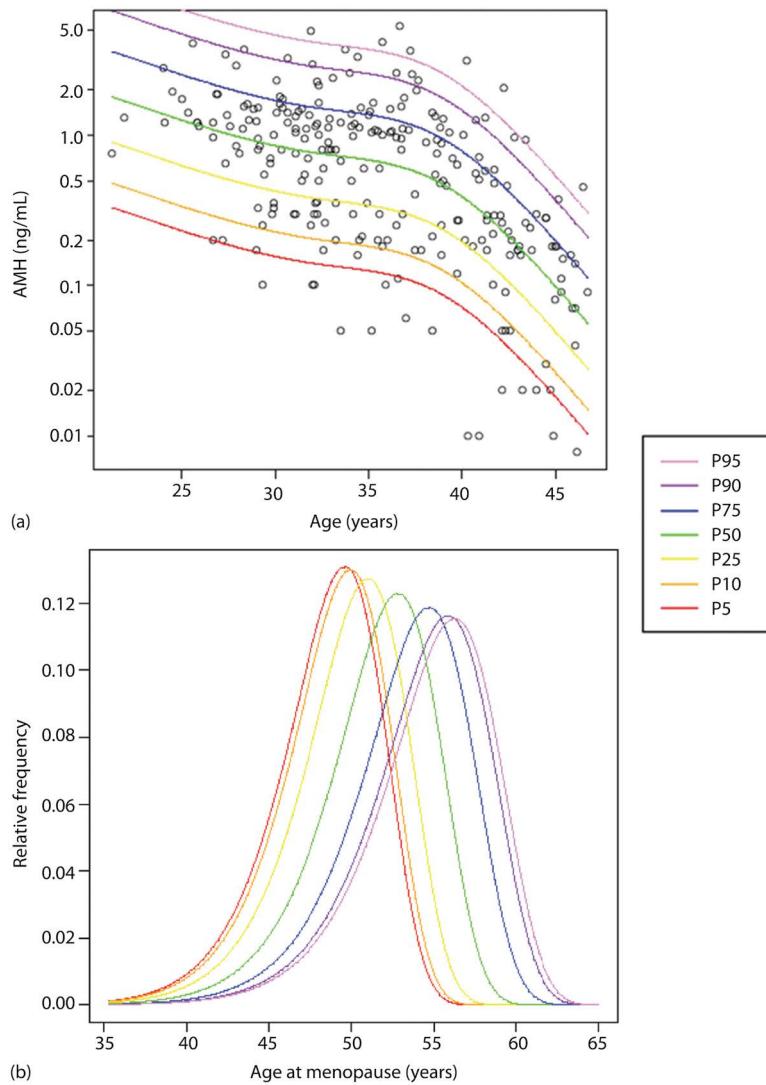


FIGURE 38.7 Nomograms for the relationship between age-specific AMH concentrations and the distribution of age at menopause. (a) The AMH levels measured at entry of the study for women at a given age are shown, measured approximately 11 years before cycle status assessment. The lines represent the upper 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.

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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

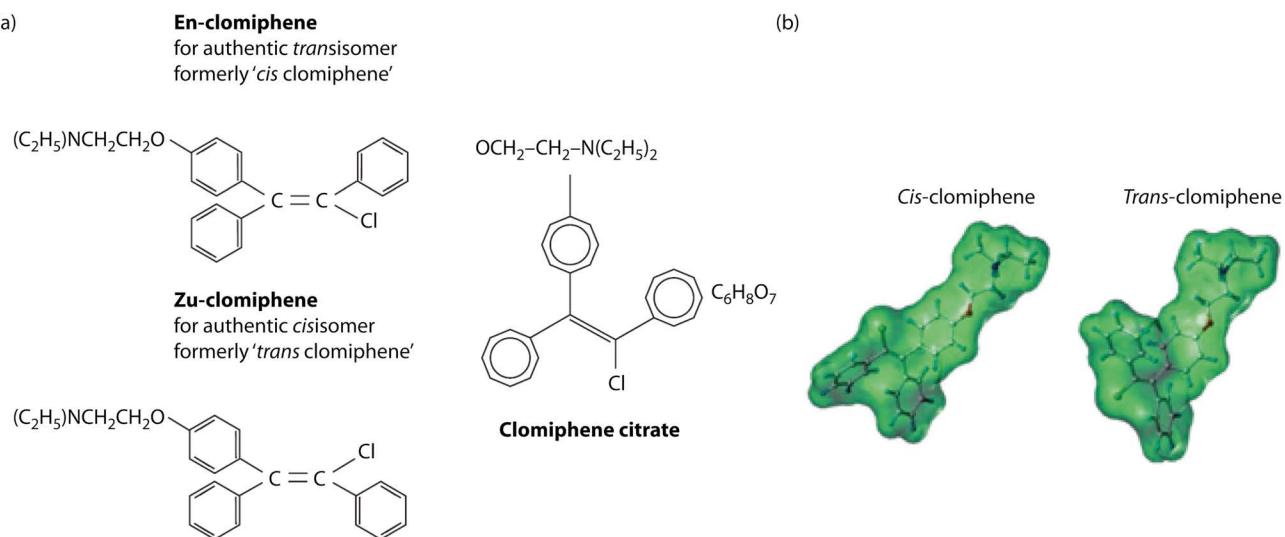


FIGURE 39.1 (a) Clomiphene citrate is available as a racemic mixture of two stereochemical isomers referred to as (*cis*) Zu-clomiphene or the (*trans*) En-clomiphene configuration, with the former being significantly more potent. In the preparations that are commercially available, the isomers are in 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.

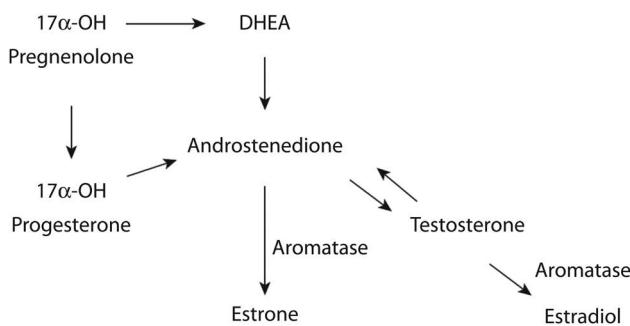


FIGURE 39.2 Aromatase inhibitor. Aromatase, an enzyme found in the liver, is responsible for the conversion of androgens—*androstenedione* and *testosterone*—into oestrogens—*oestrone* and *oestradiol*. By inhibiting aromatase, the body produces less oestrogen and maintains a higher testosterone state. 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.

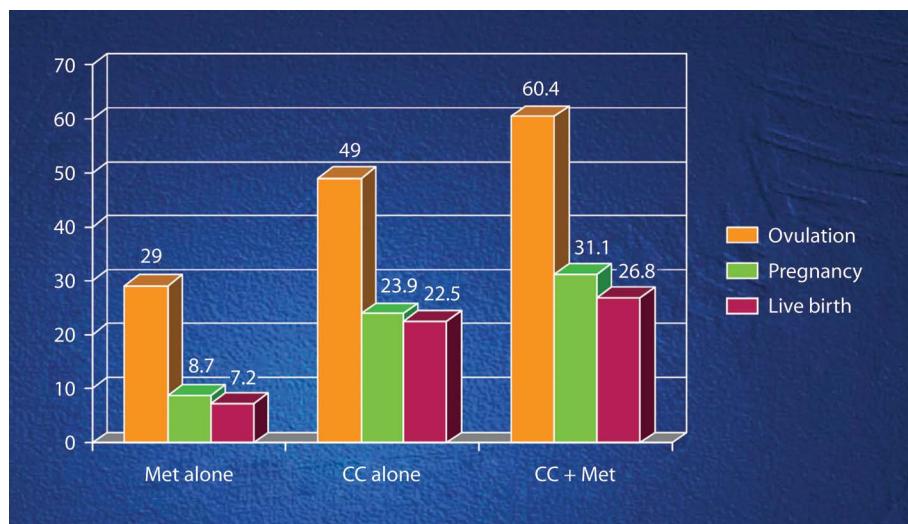


FIGURE 39.3 Ovulation, pregnancy, and live birth rates (%) in polycystic ovary syndrome patients treated with Met alone, CC alone, or Met + CC. Abbreviations: Met, metformin; CC, clomiphene citrate. (Data compiled from fig 2 of reference 68)

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].

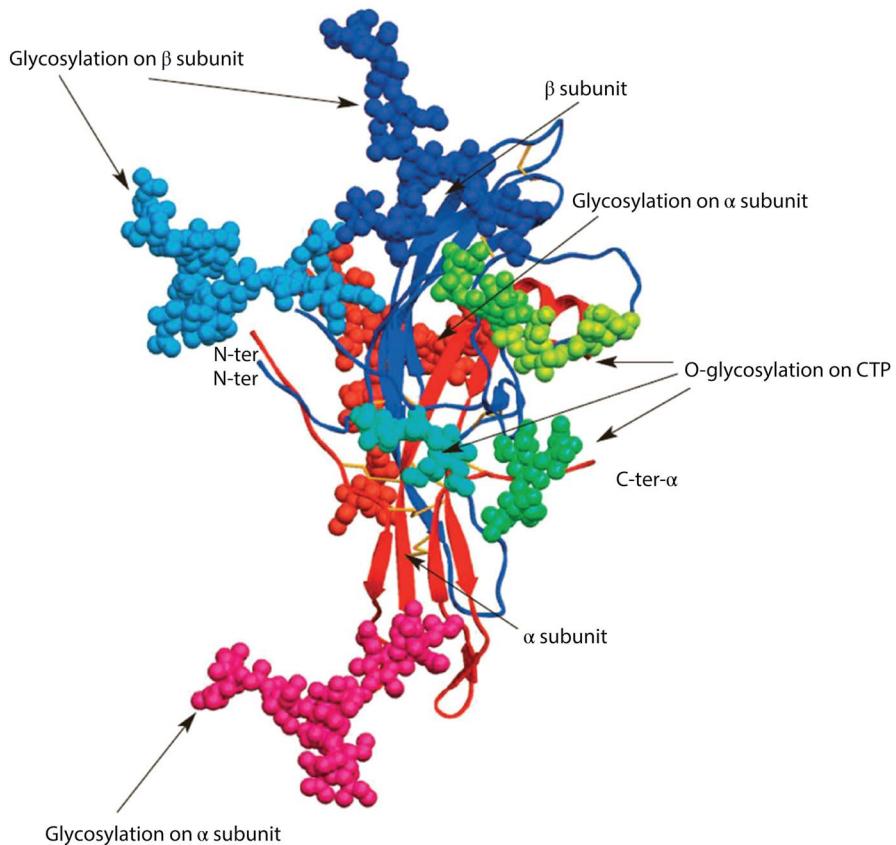


FIGURE 39.4 Human chorionic gonadotropin (hCG) model. Computerized model of hCG with full glycosylation and CTP. Abbreviation: CTP, cytidine triphosphate. (This model was created and provided by the scientific department of Serono Laboratories, United States.)

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].

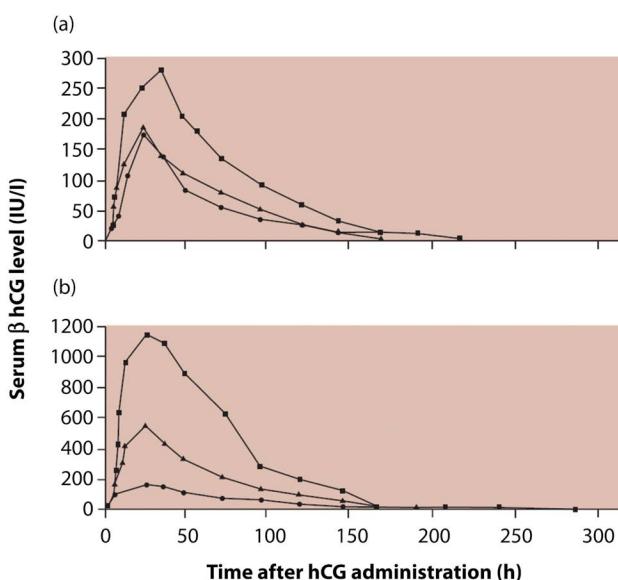


FIGURE 39.5 Pharmacokinetics of serum β -hCG in two hypogonadotropic women: (a) the first woman; (b) the second woman. Three regimens of hCG injections were applied in each woman: 10,000 IU administered subcutaneously or intramuscularly, and 5000 IU administered intramuscularly. Abbreviation: hCG, human 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

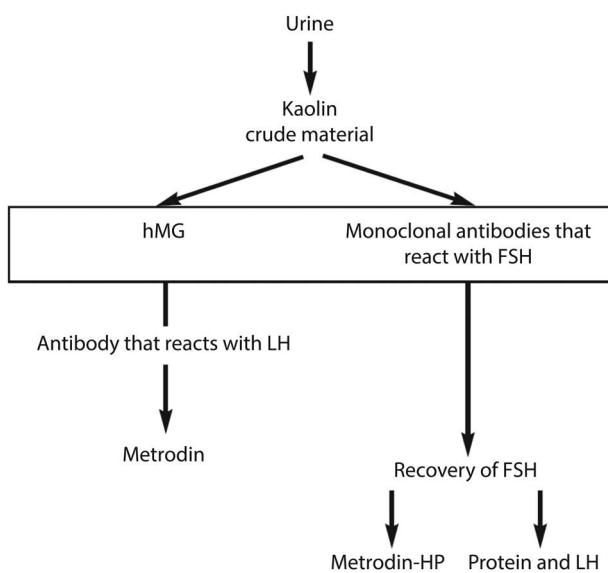


FIGURE 39.6 Schematic presentation of the production of hMG and the purification of urinary FSH and HP-FSH. Abbreviations: FSH, follicle-stimulating hormone; hMG, human menopausal gonadotropin; HP, high-purity; LH, luteinizing hormone.

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
	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.

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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.

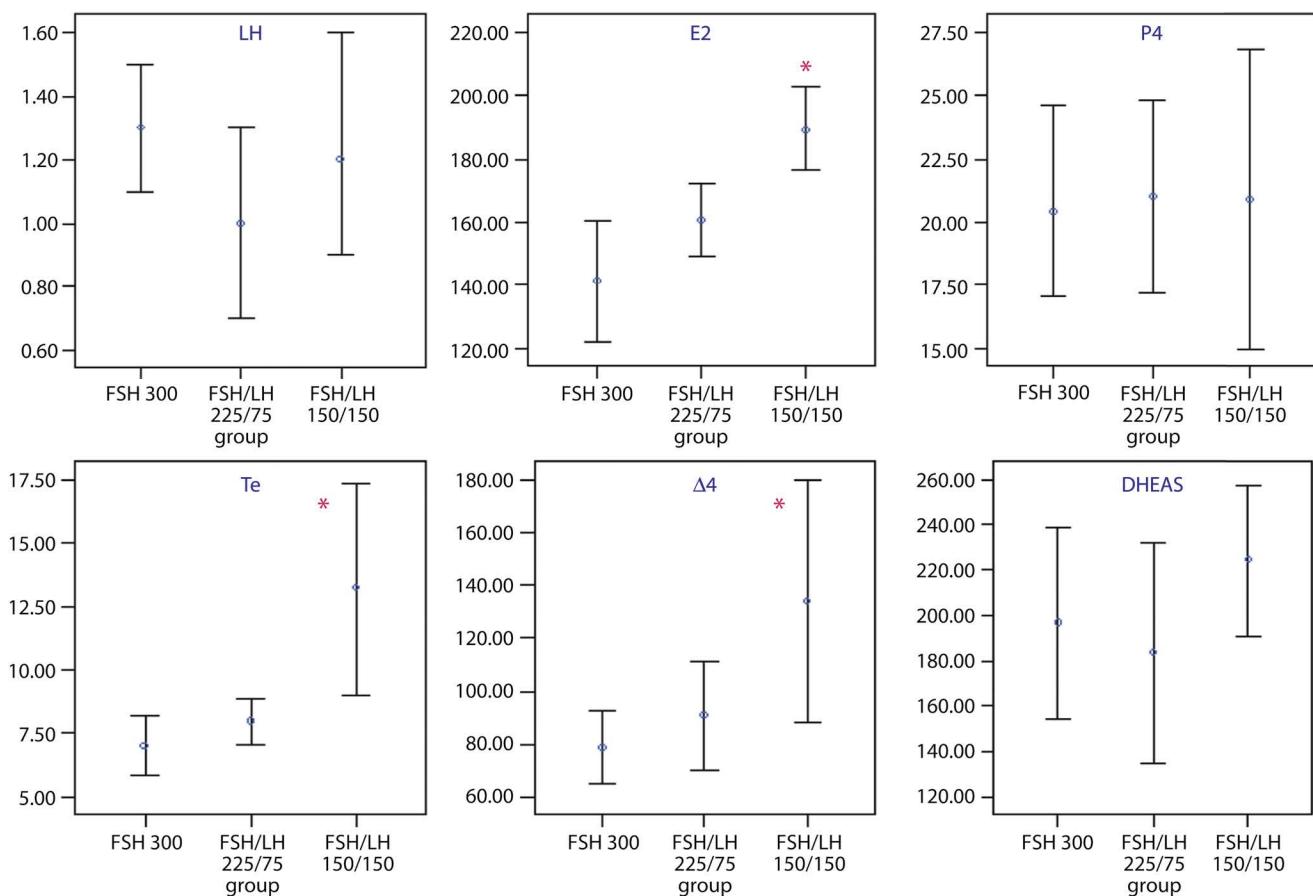


FIGURE 40.1 Follicular fluid hormonal determinations on the day of human chorionic gonadotropin observation. Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone (mIU/mL); E2, oestradiol (pg/mL); DHEAS, dehydroepiandrosterone sulphate (mcg/dL); P4, progesterone (ng/mL); Te, testosterone (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].

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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

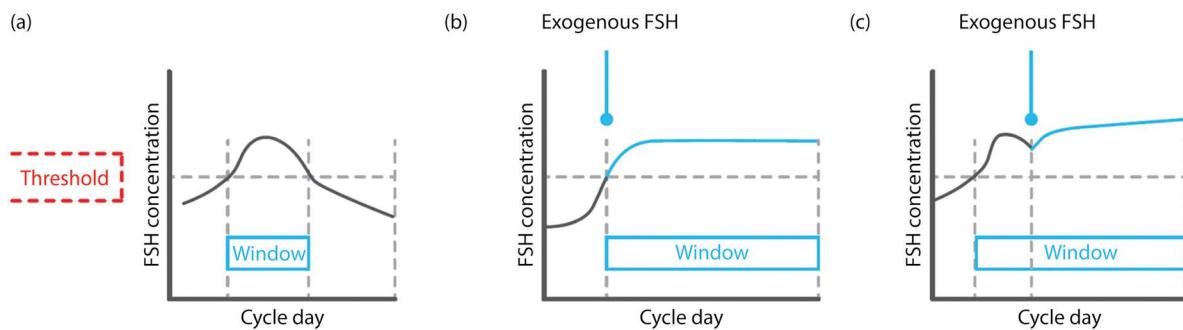


FIGURE 41.1 The FSH window corresponds to the period during which FSH levels are above the threshold levels for continuing antral follicle growth. (a) Natural menstrual cycle; (b) long GnRH agonist protocol; (c) GnRH antagonist cycle. Abbreviation: FSH, follicle-stimulating hormone.

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).

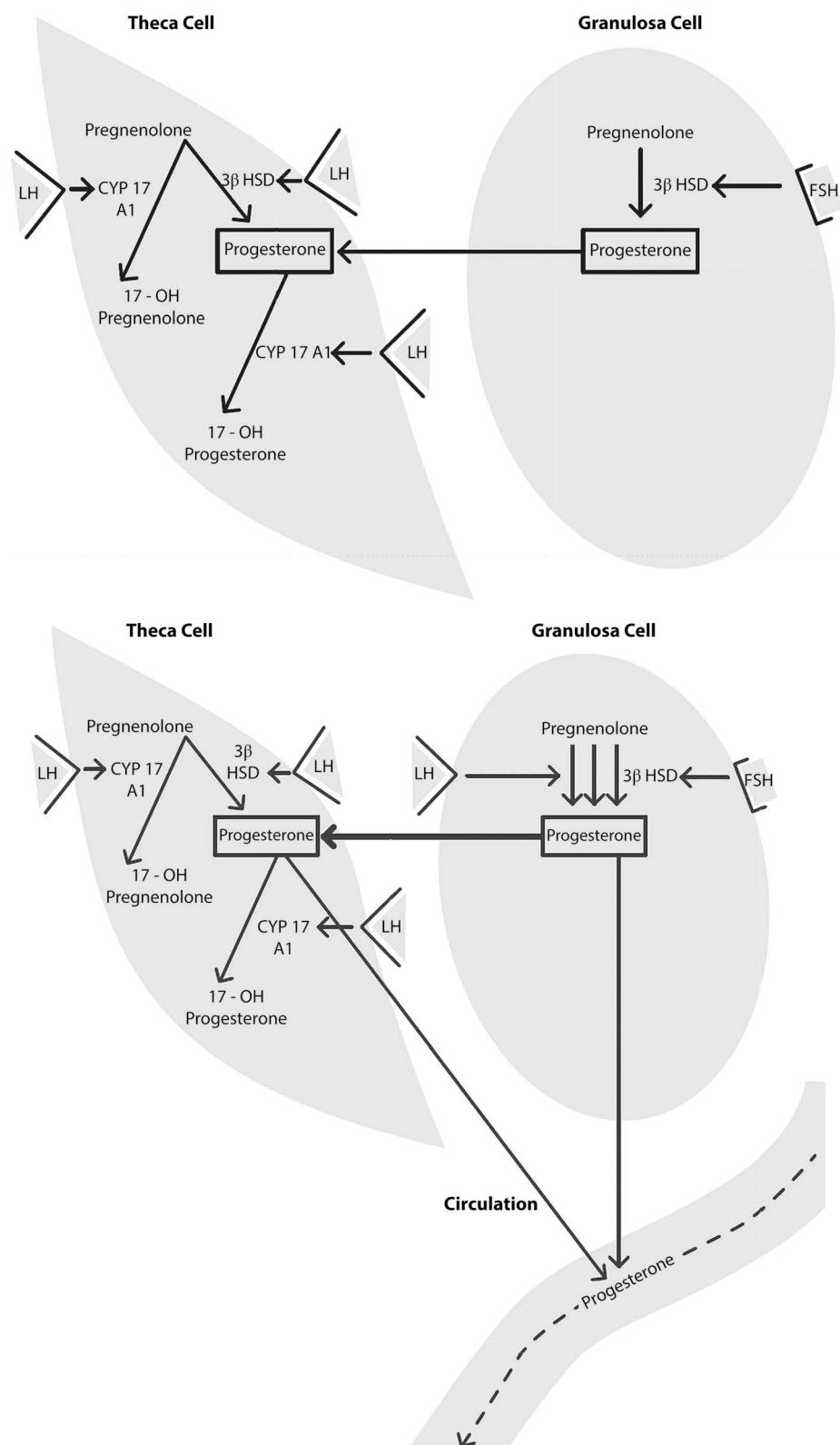


FIGURE 41.2 Progesterone synthesis and metabolism during the follicular phase. CYP 17 A1 (C17 hydroxylase) activity is only present in theca cells and hence progesterone produced by the granulosa cells diffuses into the theca cells to be hydroxylated. (a) During the early follicular phase, LH acts only on theca cells to stimulate 3β 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

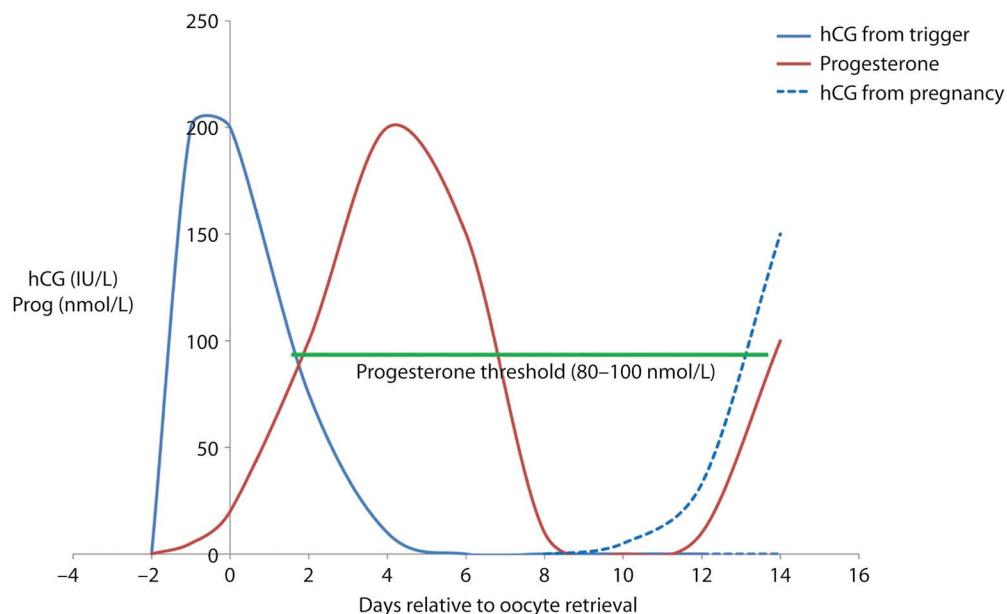


FIGURE 41.3 Circulating hCG and progesterone levels from hCG administration until early pregnancy during an *in vitro* fertilization cycle (1 nmol/L progesterone equals 0.31 ng/mL). Abbreviation: hCG, human chorionic gonadotropin.

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.

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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.

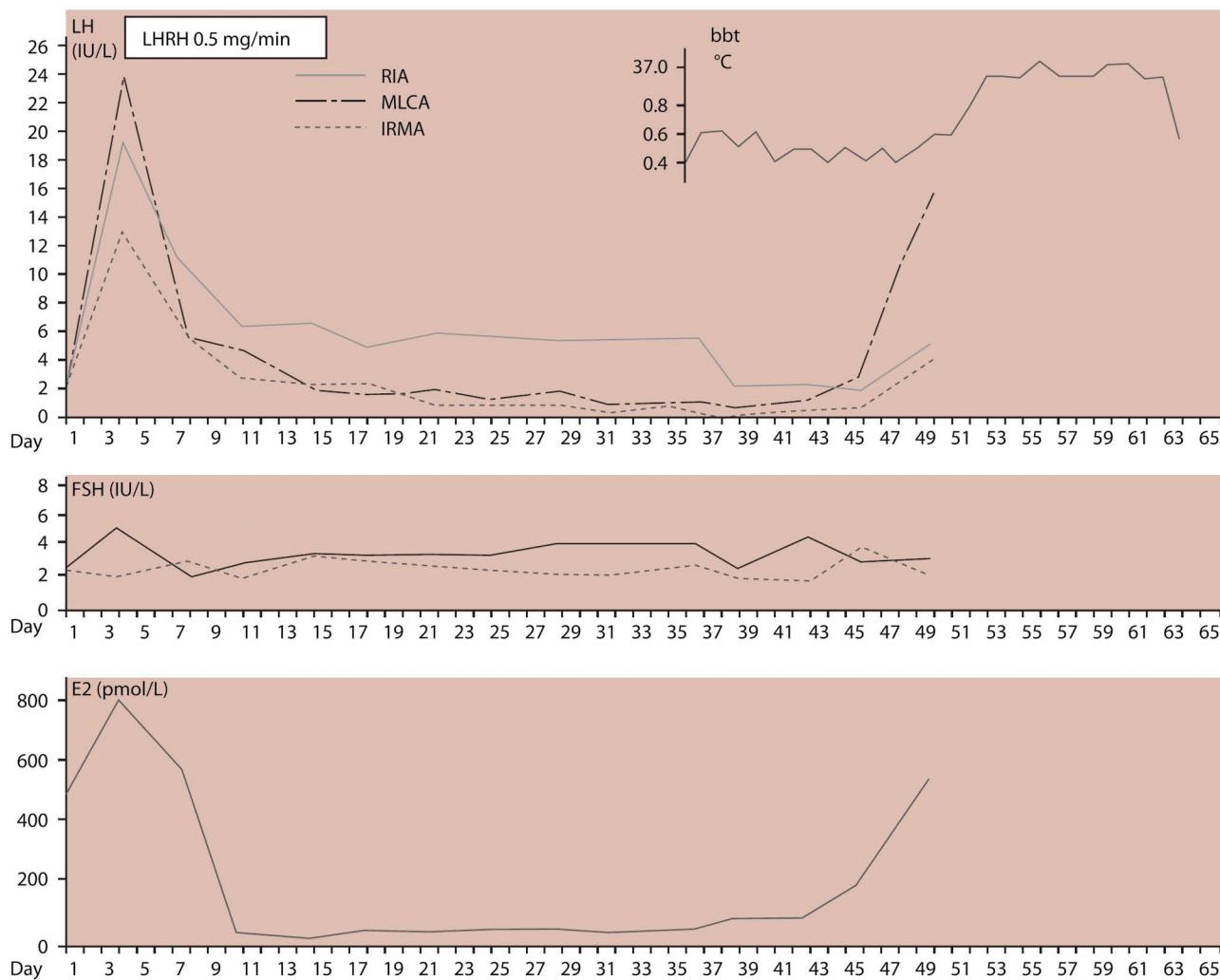


FIGURE 42.1 Hormone levels for LH, FSH, and E2 in a patient with continuous intravenous infusion of 0.5 mg/minute LHRH. LH was measured with three different assays and FSH with two different assays. Abbreviations: RIA, radioimmunoassay; MLCA, Magic Lite chemiluminescence assay; IRMA, immunoradiometric 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			
	1	2	3	4	5	6	7	8	9	10
Amino acid no										
Native GnRH	Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	GlyNH ₂
Nonapeptides										
Leuprolide (Lupron, Lucrin), buserelin (Suprefact), goserelin (Zoladex), histrelin (Suprelrin), 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).

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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

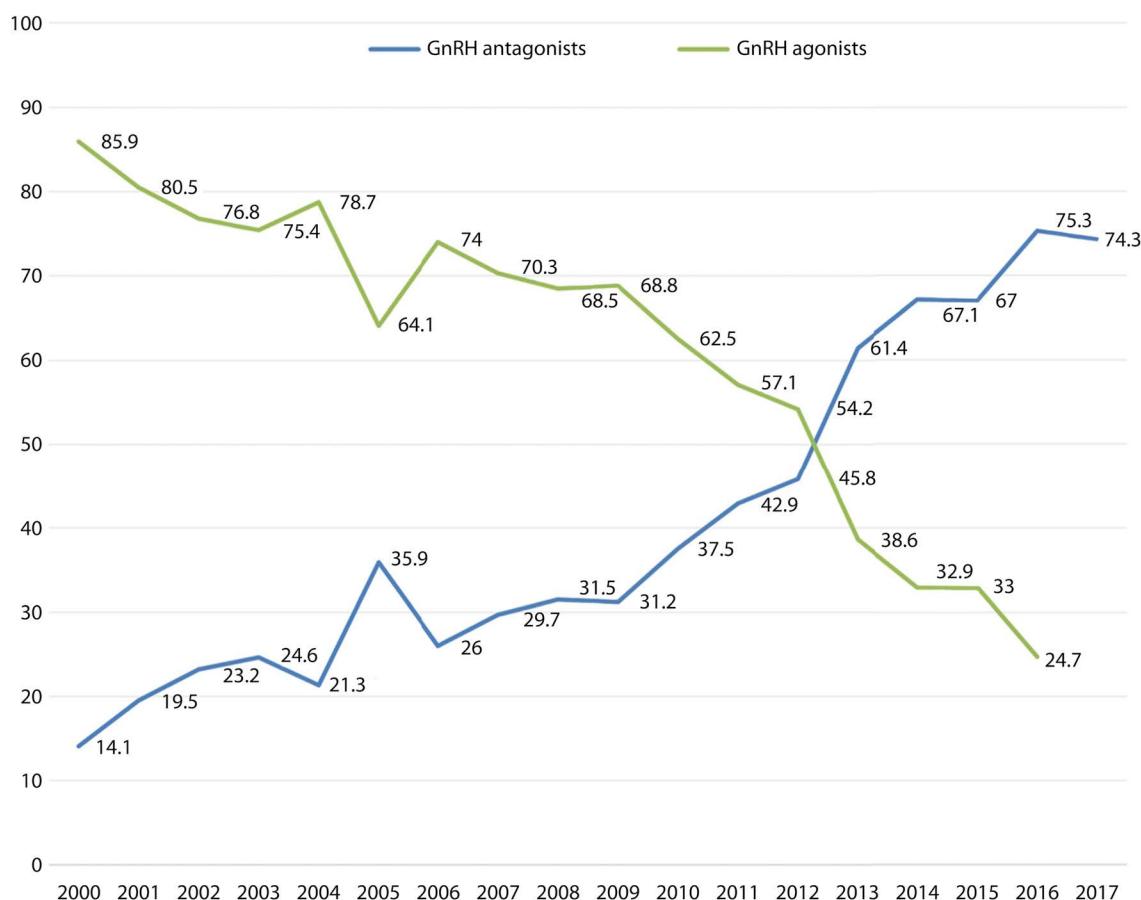


FIGURE 43.1 Proportion of cycles performed with either GnRH agonists or antagonists based on data published by the Deutsches IVF Register (DIR) (2000–2017). Abbreviation: GnRH, gonadotropin-releasing hormone.

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,

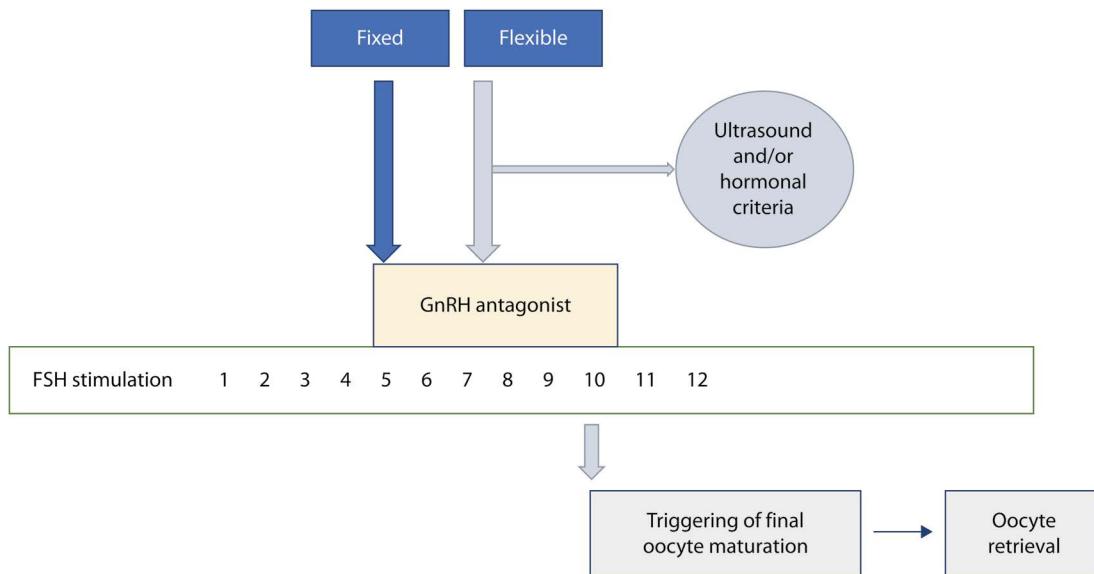


FIGURE 43.2 GnRH antagonist protocols. Fixed day 5 protocol: initiation of GnRH antagonist on day 5 of stimulation daily and continuation until the day of triggering final oocyte maturation. Flexible dose protocol: initiation of GnRH antagonist after certain endocrine and/or sonographic criteria indicating 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

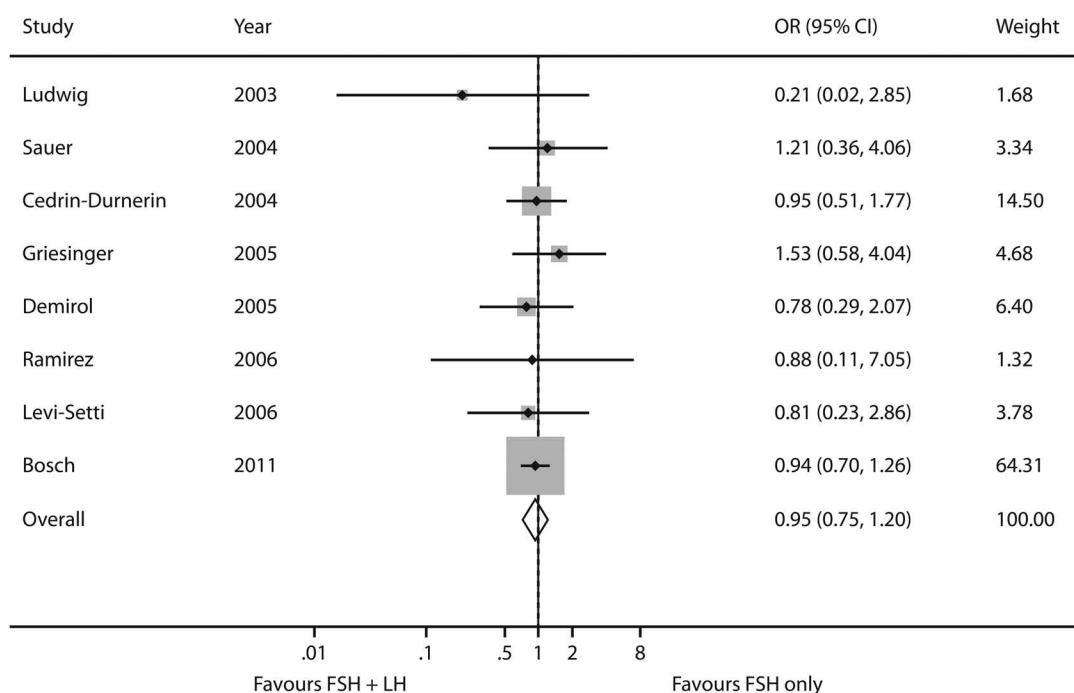


FIGURE 43.3 Addition of recombinant LH to recombinant FSH in gonadotropin-releasing hormone antagonist cycles. Abbreviations: CI, confidence interval; FSH, follicle-stimulating hormone; LH, luteinizing hormone; OR, odds ratio. (Based on [61].)

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.

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44

GnRH AGONIST TRIGGERING

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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

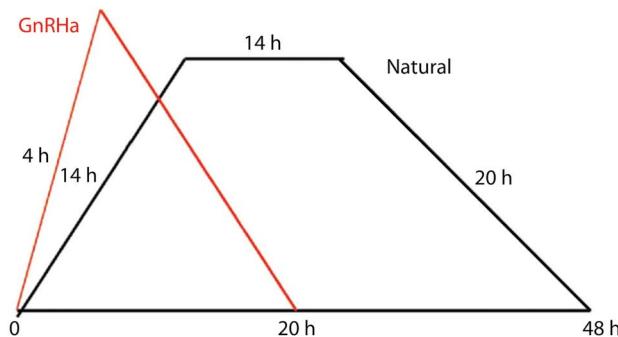


FIGURE 44.1 Luteinizing hormone surge in a natural cycle and after GnRH agonist trigger. (From [141], with permission.)

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

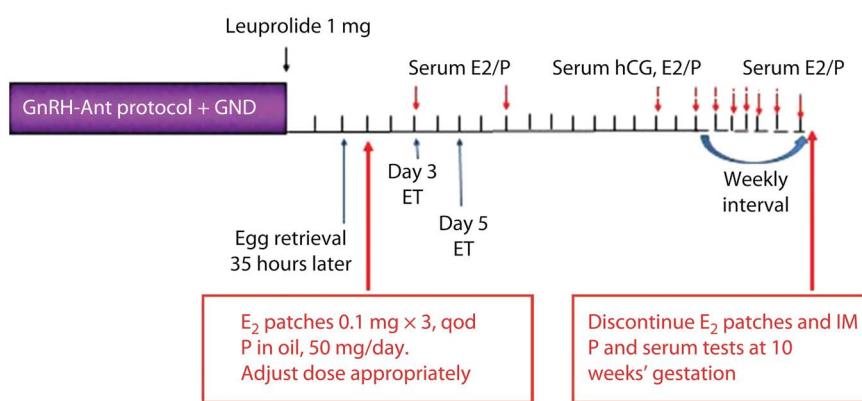


FIGURE 44.2 Components of intensive luteal phase support. E2 = estradiol, P = progesterone, IM = intramuscular, qod = every other day

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

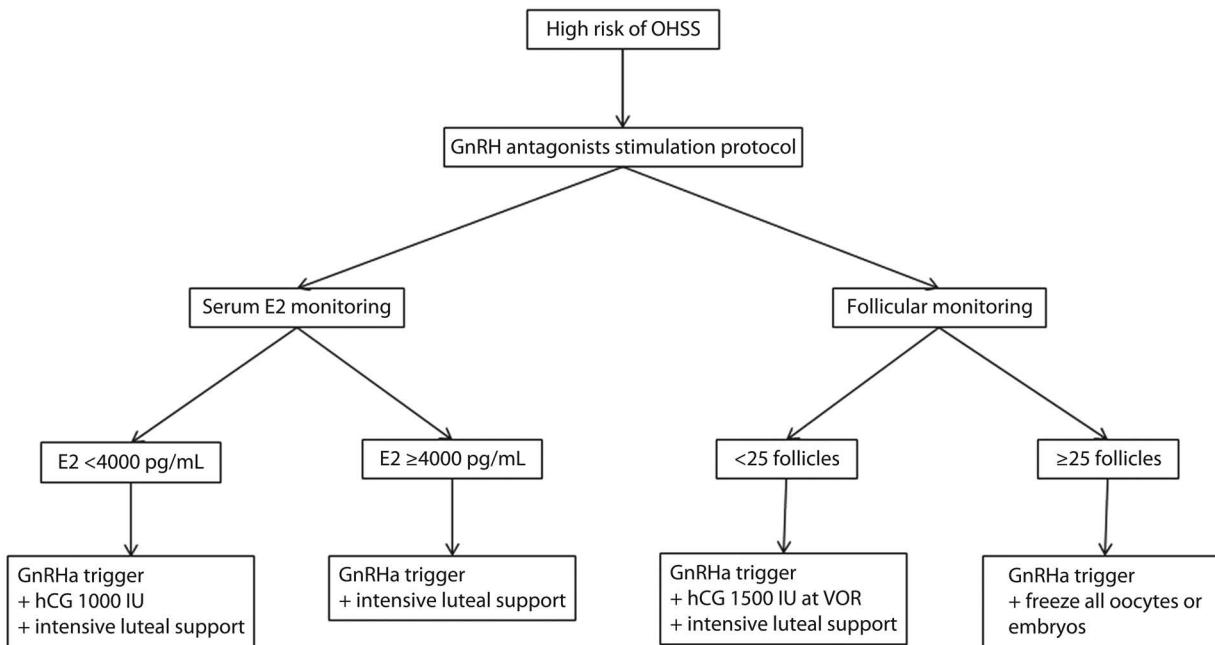


FIGURE 44.3 Suggested luteal-phase support protocols by high-responder characteristics. VOR = vaginal oocyte retrieval

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.

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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%

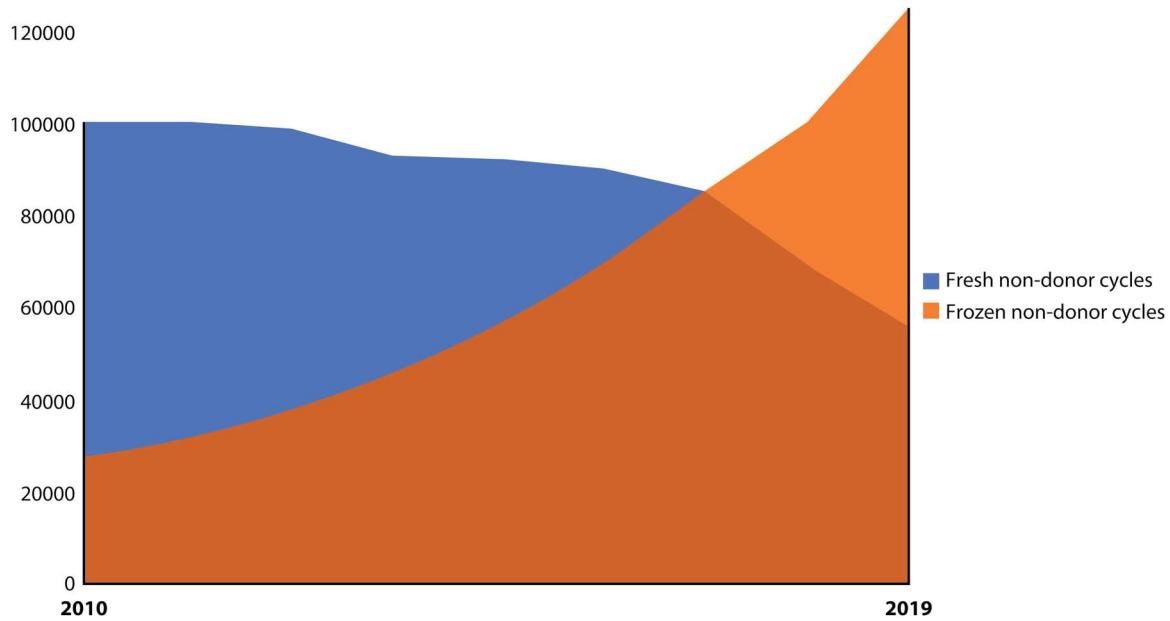


FIGURE 45.1 Number of non-donor ART cycles. (Data from Centers for Disease Control and Prevention, ART success rates, 2010–2019; available at <https://www.cdc.gov/art/reports/archive.html>)

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.

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CONTROLLED OVARIAN STIMULATION FOR FREEZE-ALL CYCLES

Yossi Mizrachi and Ariel Weissman

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.

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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,

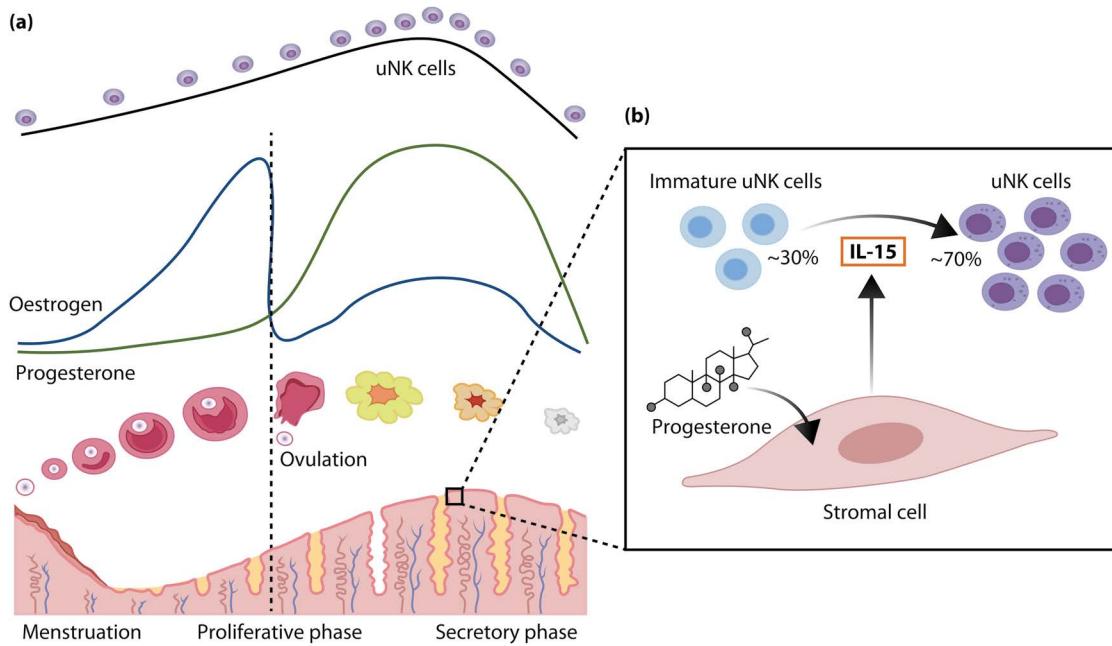


FIGURE 47.1 (a) uNK cells throughout the menstrual cycle. The increase this population suffers during the proliferative phase can be observed, along with the subsequent reduction if fertilization does not occur. (b) Proposed mechanism of progesterone-dependent uNK cell maturation and proliferation [7].

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

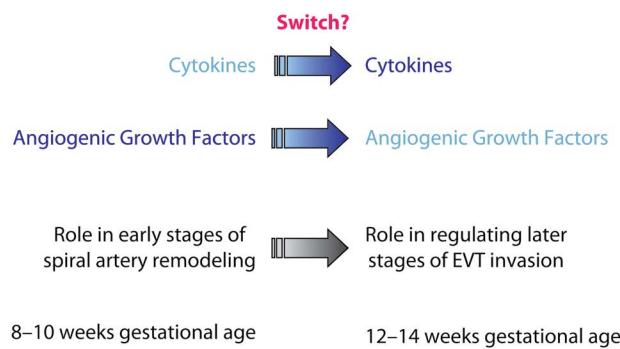


FIGURE 47.2 Schematic of the differences in uNK cells functions and secretions with gestational age. Since 12 weeks of pregnancy, these cells start to produce more cytokines implicated in the regulation of EVT invasion and reduce the secretion of angiogenic factors [55].

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

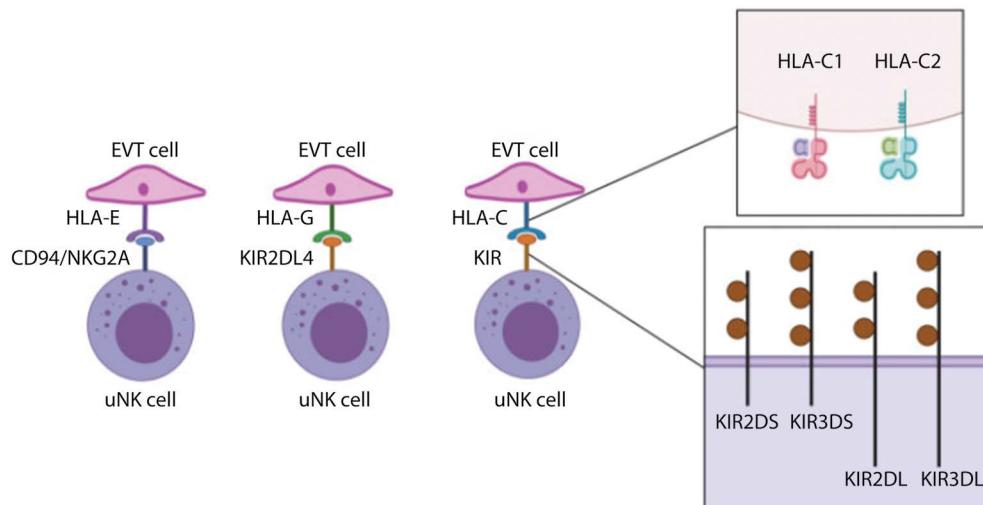


FIGURE 47.3 Interactions between uNK cells and HLA ligands from EVT cells. Isoforms of HLA-C are also presented, as well as KIR classification, depending on the number of extracellular domains and tail length [7].

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

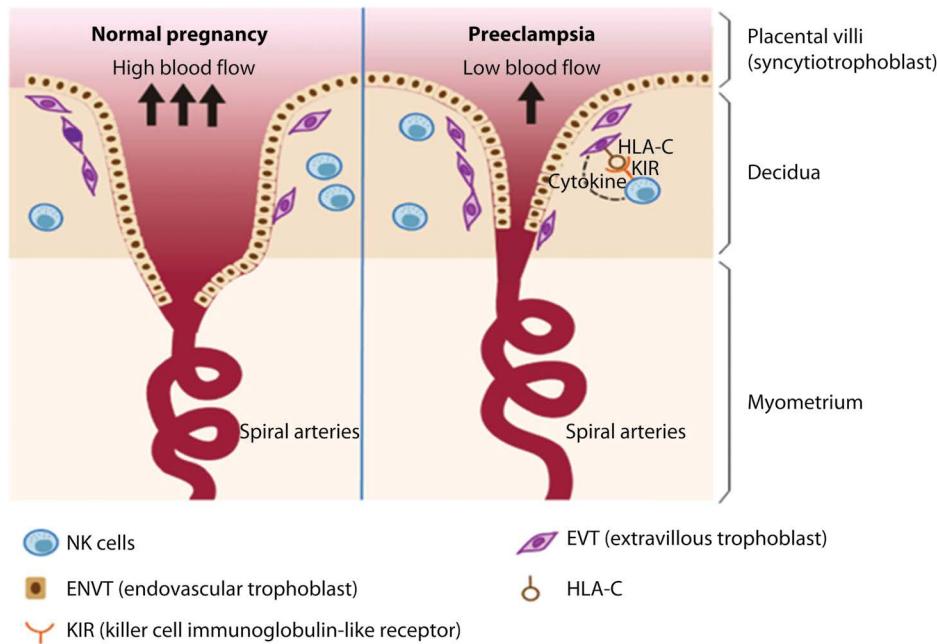


FIGURE 47.4 Schematic representation of normal (left picture) and poor placentation (right picture) during early pregnancy [53].

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]	

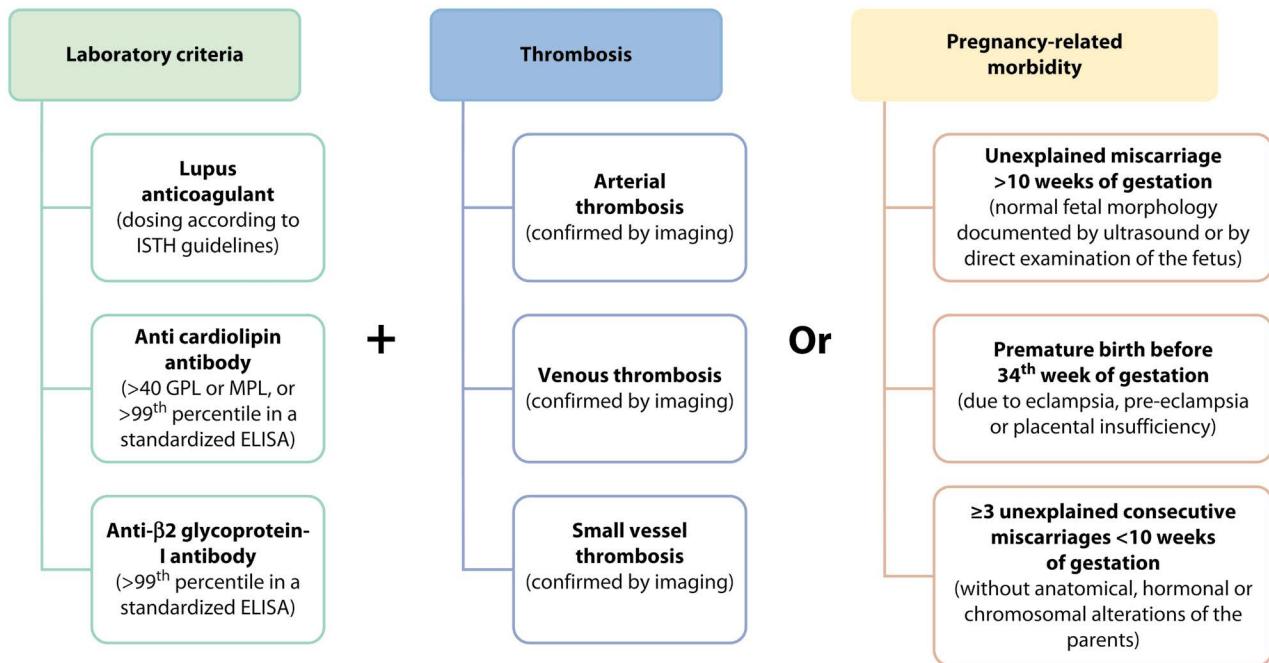


FIGURE 47.5 Summary of criteria for antiphospholipid syndrome (APS) diagnosis according to Sapporo criteria. Abbreviations: ISTH: International Society of Thrombosis and Haemostasis. GPL: Glycopeptidolipid; MPL: Monophosphoryl lipid A [90].

(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 preconceptual 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.

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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.

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HOME MONITORING OF ART CYCLES

Wenjing Zheng, Jan Gerris, Eline Dancet, and Thomas D'Hooghe

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

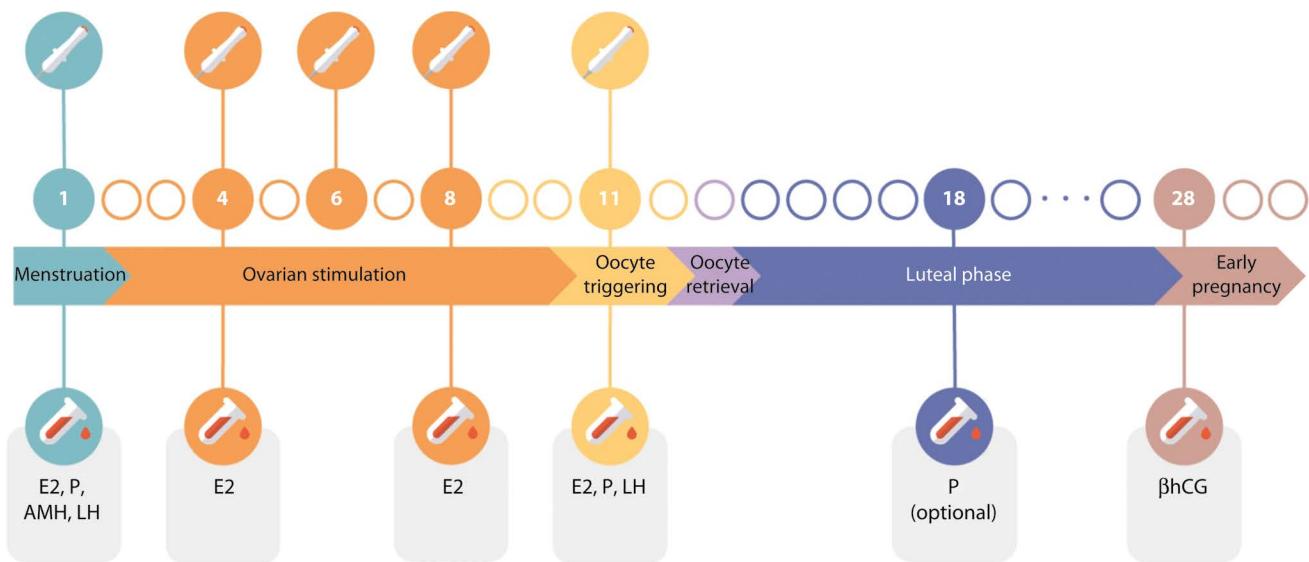


FIGURE 49.1 Typical schedule for the frequency of transvaginal ultrasound (top panel) and serum hormonal monitoring (bottom panel) during a cycle of COS for ART. The numbers in the circles represent the days in a typical stimulation cycle corresponding to the respective phases (only days on which monitoring 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.

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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**.

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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.

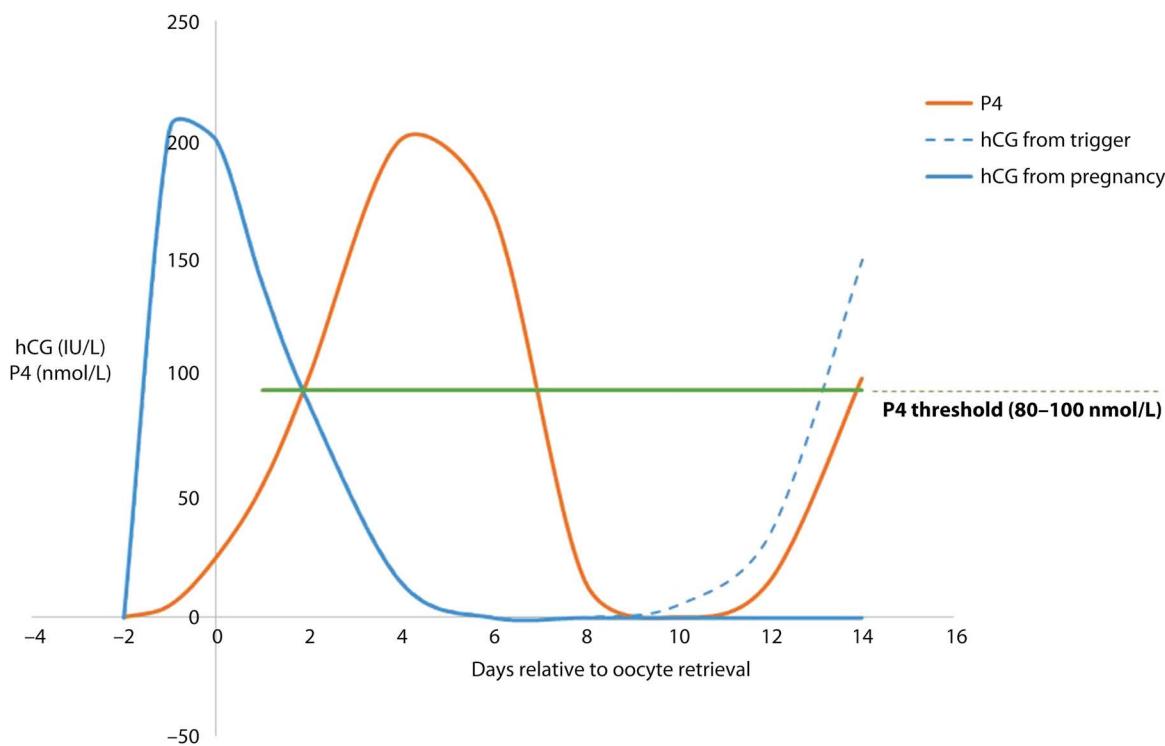


FIGURE 51.1 Representation of hCG and P4 levels from the day of hCG trigger until early pregnancy during an IVF stimulated cycle. Serum P4 levels are represented in nmol/L. (Adapted from [22].)

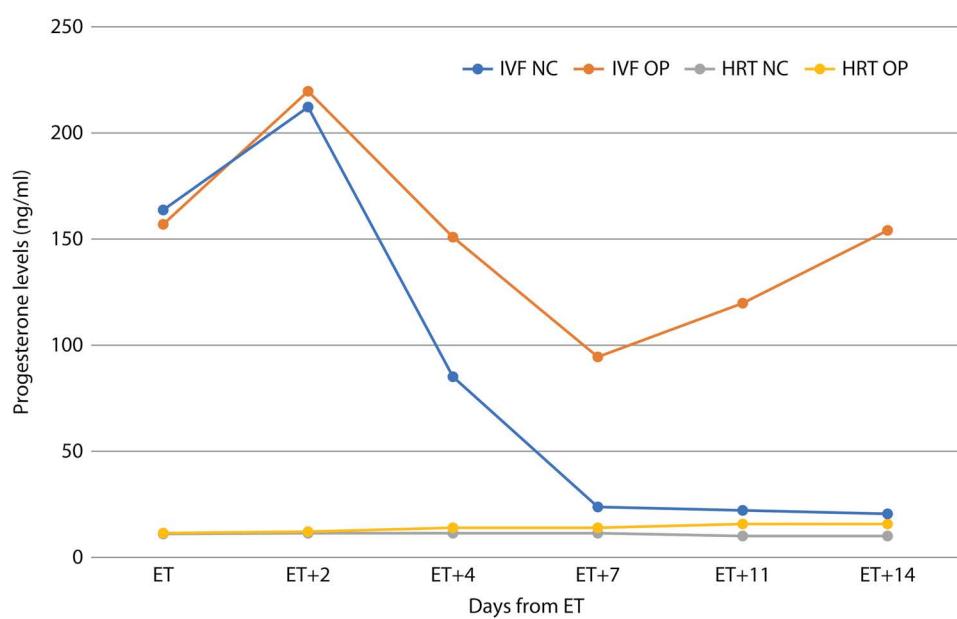


FIGURE 51.2 Progesterone behaviour from embryo transfer (ET) day onwards in IVF cycles versus HRT cycles, discriminating by non-conception (NC) and ongoing pregnancies (OP) and all of them using LPS. This figure was created considering data from Sonntag et al. for the IVF cycle [30] and Labarta et al. 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 25.2–31.4 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

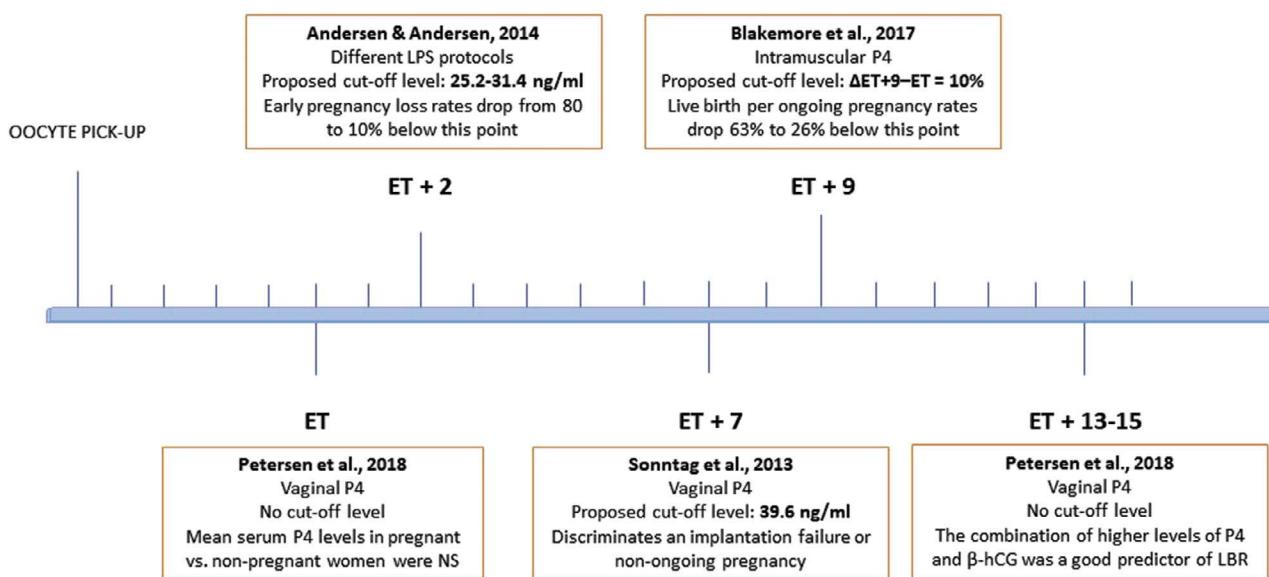


FIGURE 51.3 Representation of the different serum progesterone cut-off levels proposed by different studies regarding success rates throughout the luteal phase in IVF cycles. Abbreviations: NS, non-significant; ET, embryo transfer; P4, progesterone; LPS, luteal phase support; LBR, live birth rate; OPR, 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.

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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

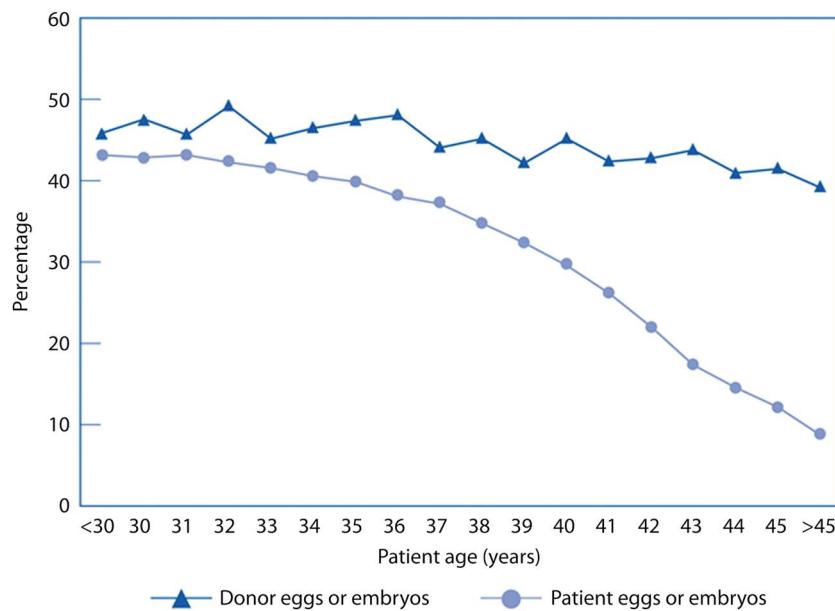


FIGURE 52.1 Percentage of embryo transfers that resulted in live birth delivery, by patient age and egg or embryo. (United States, Centers for Disease Control and Prevention data, 2019 [9].)

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

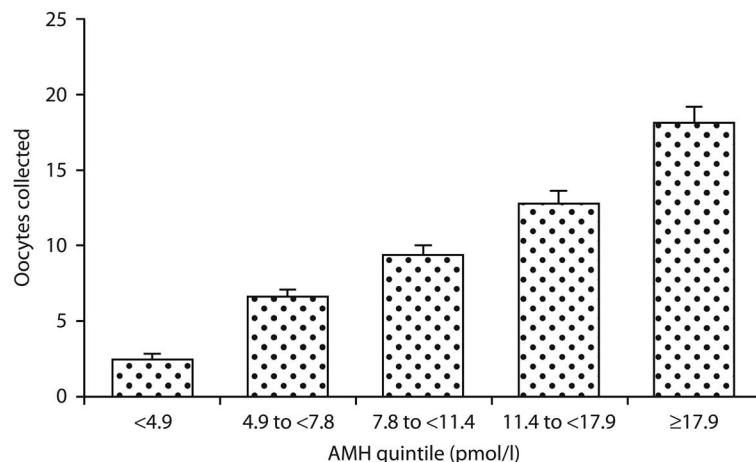


FIGURE 52.2 Mean oocyte yield per AMH quintile. Values are mean \pm standard error of the mean (SEM) [41]. Abbreviation: AMH, anti-Müllerian hormone.

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,

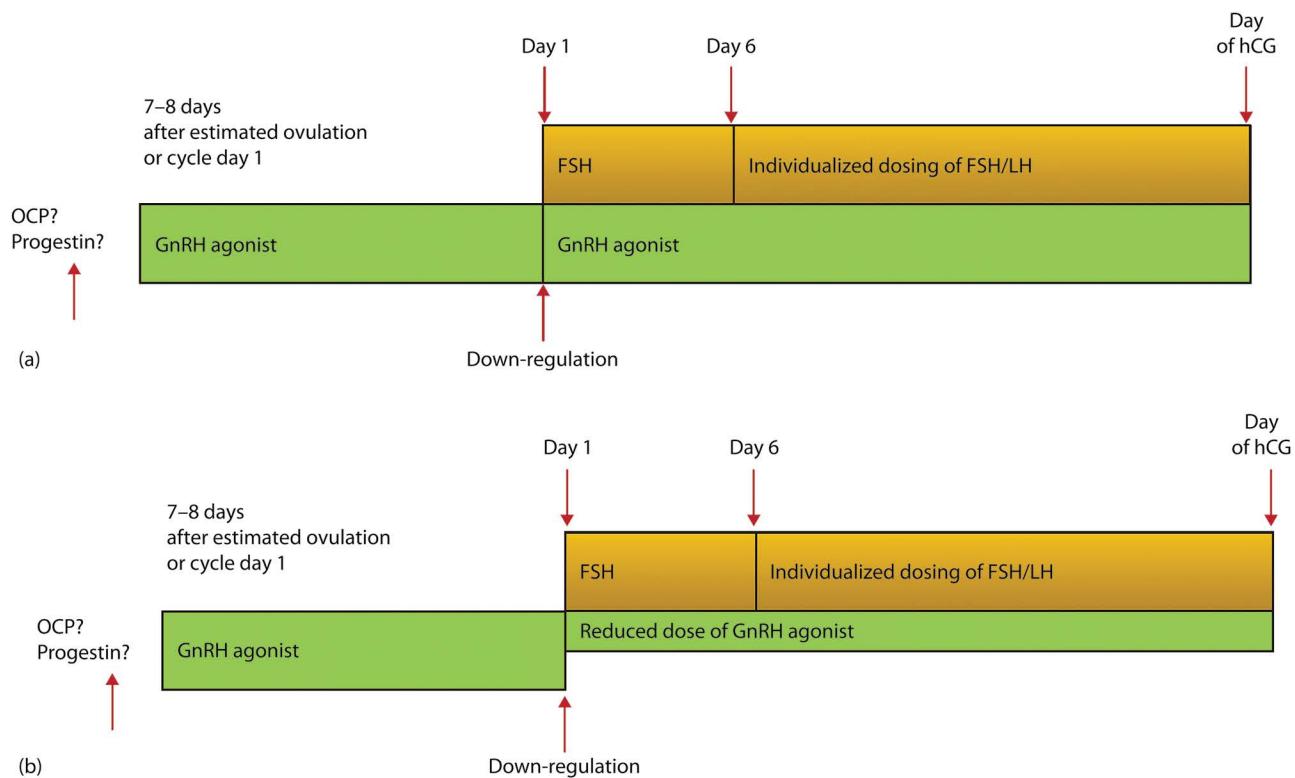


FIGURE 52.3 (a) The long GnRH agonist protocol. (b) The “mini-dose” long agonist protocol. Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; OCP, oral contraceptive pill.

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 \geq 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).

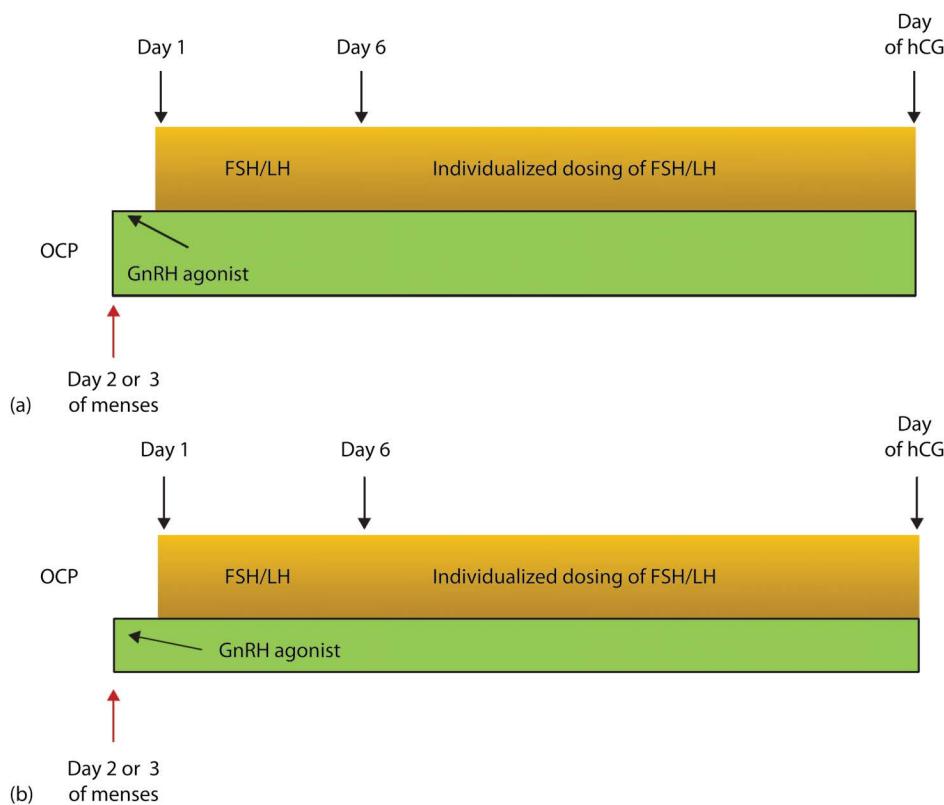


FIGURE 52.4 (a) The short GnRH agonist protocol. (b) The “microdose” flare GnRH agonist protocol. Abbreviations: FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; OCP, oral contraceptive pill.

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

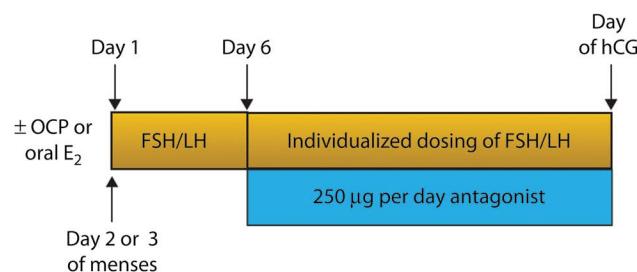


FIGURE 52.5 Gonadotropin-releasing hormone antagonist protocol. Abbreviations: E2, oestradiol; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; OCP, oral contraceptive pill.

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	Cetralrelax, 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	Cetralrelax, 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	Cetralrelax, 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	Cetralrelax/ 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

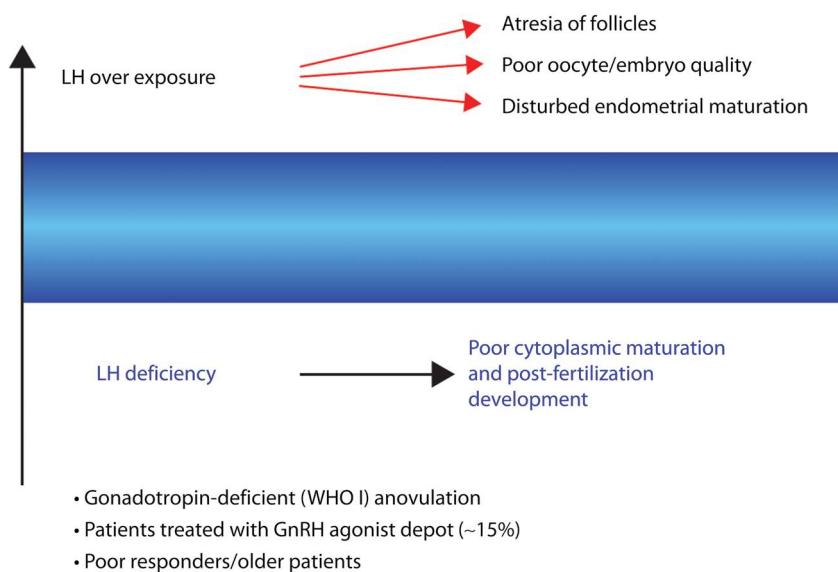


FIGURE 52.7 The LH therapeutic window concept. Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; WHO, World Health Organization.

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 17β -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.

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THE POSEIDON STRATIFICATION OF “LOW-PROGNOSIS PATIENTS IN ART”

Management Strategies and Outcomes

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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.

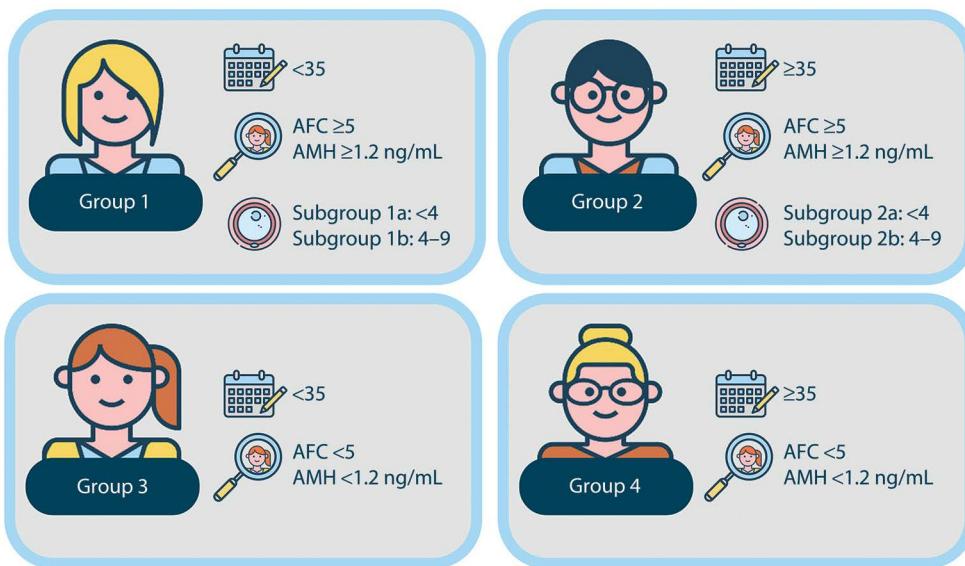


FIGURE 53.1 POSEIDON criteria. Four distinct groups of low-prognosis patients can be established based on quantitative and qualitative parameters, namely, (i) the age of the patient and its related embryo aneuploidy rate; (ii) ovarian biomarkers (antral follicle count [AFC] and/or anti-Müllerian hormone [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

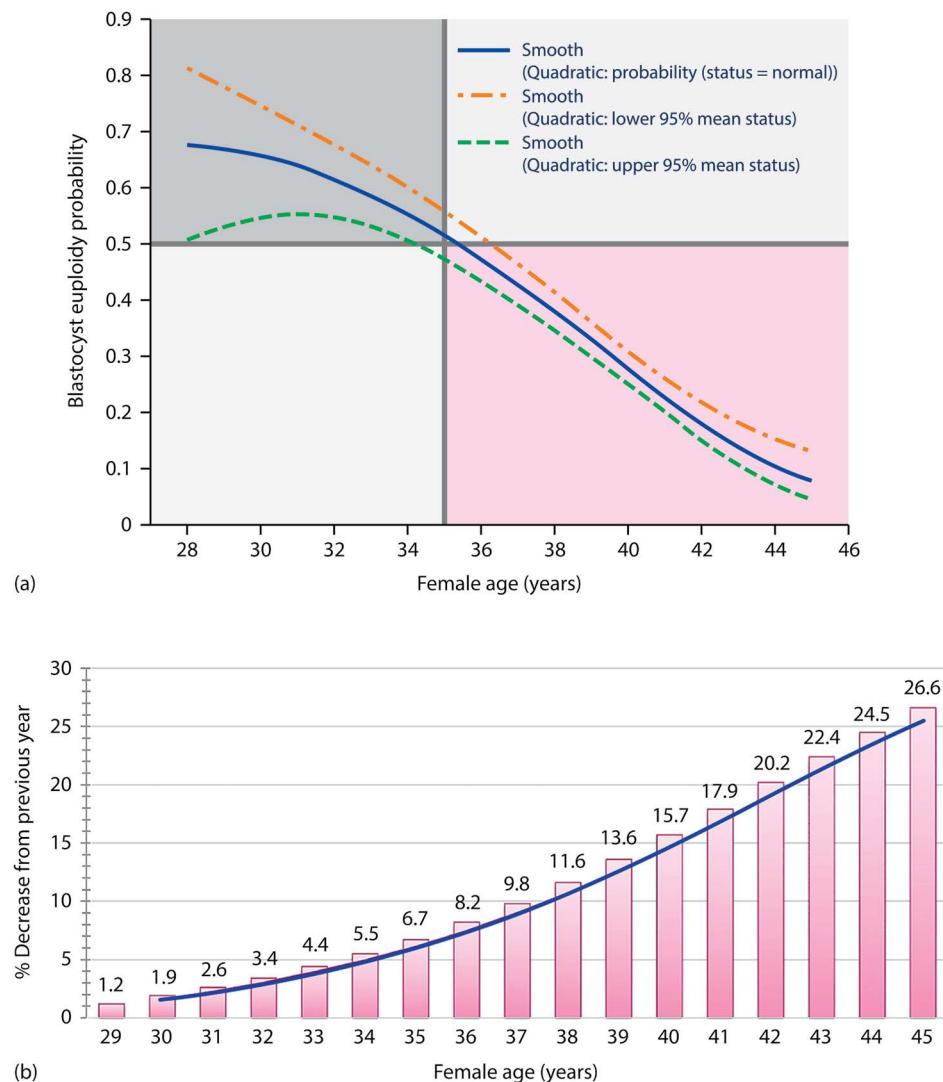


FIGURE 53.2 Impact of female age on blastocyst euploidy. Logistic regression analysis of 1220 trophectoderm biopsies from 436 infertile couples undergoing ICSI and pre-implantation genetic testing for aneuploidy (PGT-A) by next-generation sequencing. The dependent and independent variables were embryo 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.

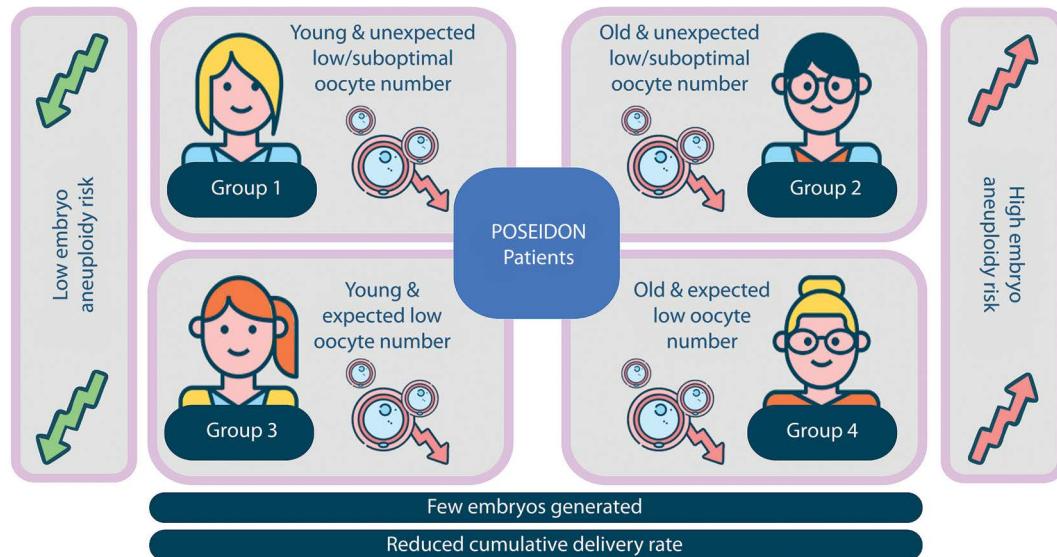


FIGURE 53.3 Snapshot of POSEIDON's criteria clinical significance. Groups 1 and 3 patients are young, and, consequently, their risk of embryo aneuploidy is relatively low. Patients of groups 2 and 4 have advanced maternal age, and, as a result, their risk of embryo aneuploidy is increased. The bottom 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

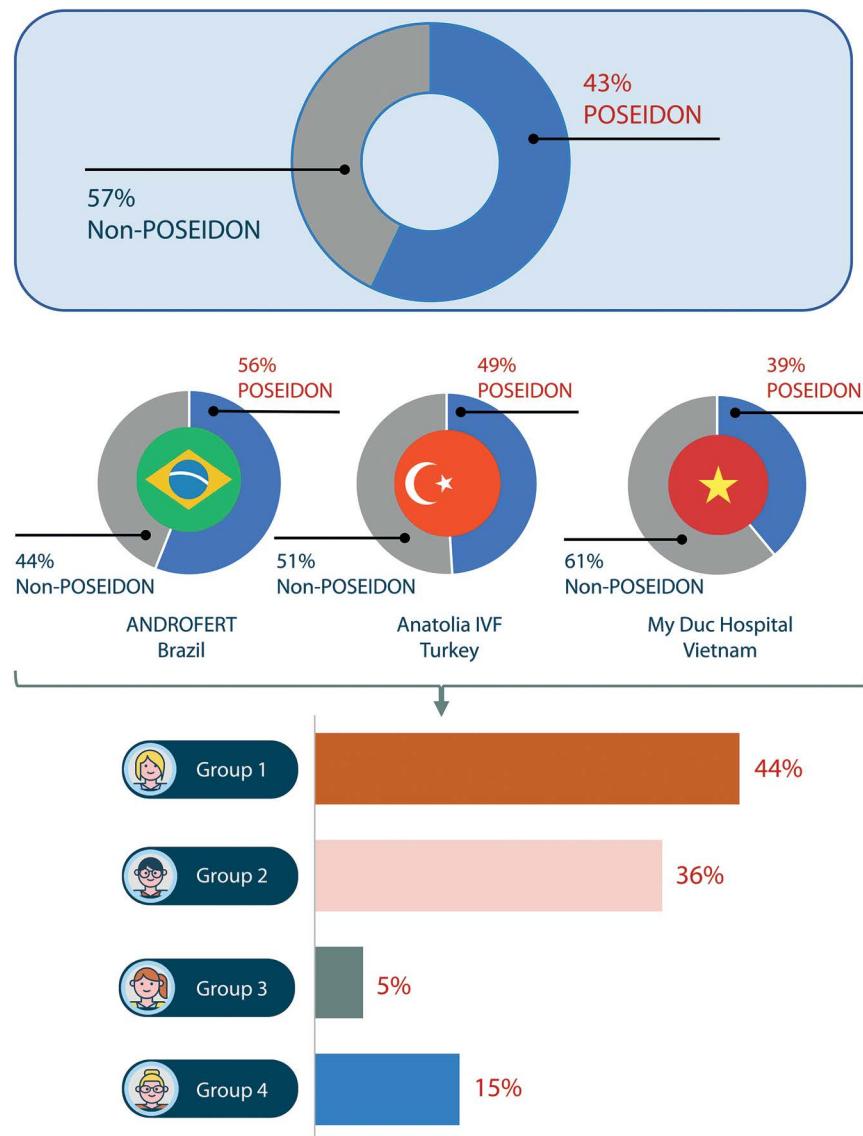


FIGURE 53.4 Prevalence of low-prognosis patients under the POSEIDON criteria. Data from a multicentre population-based study of 13,146 patients showed POSEIDON patients represented 42.9% (95% confidence interval [CI] 42.0–43.7) of the studied population, and the prevalence rates varied across study centres. (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%)

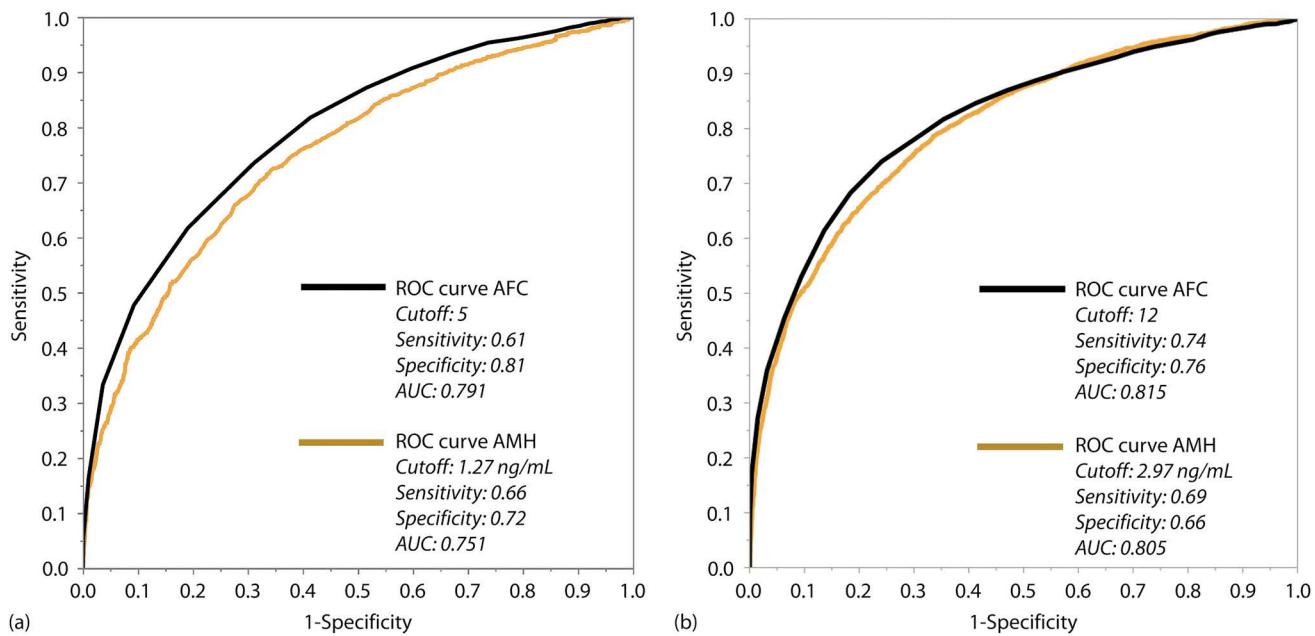


FIGURE 53.6 Validation of POSEIDON criteria biomarkers' thresholds. The figure shows the age-adjusted ROC curves of AFC and AMH to predict low (fewer than four) and suboptimal (four to nine) oocyte yields. (a) For low oocyte yield, the optimal AFC cut-off value was 5, with a sensitivity of 0.61, a specificity 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?

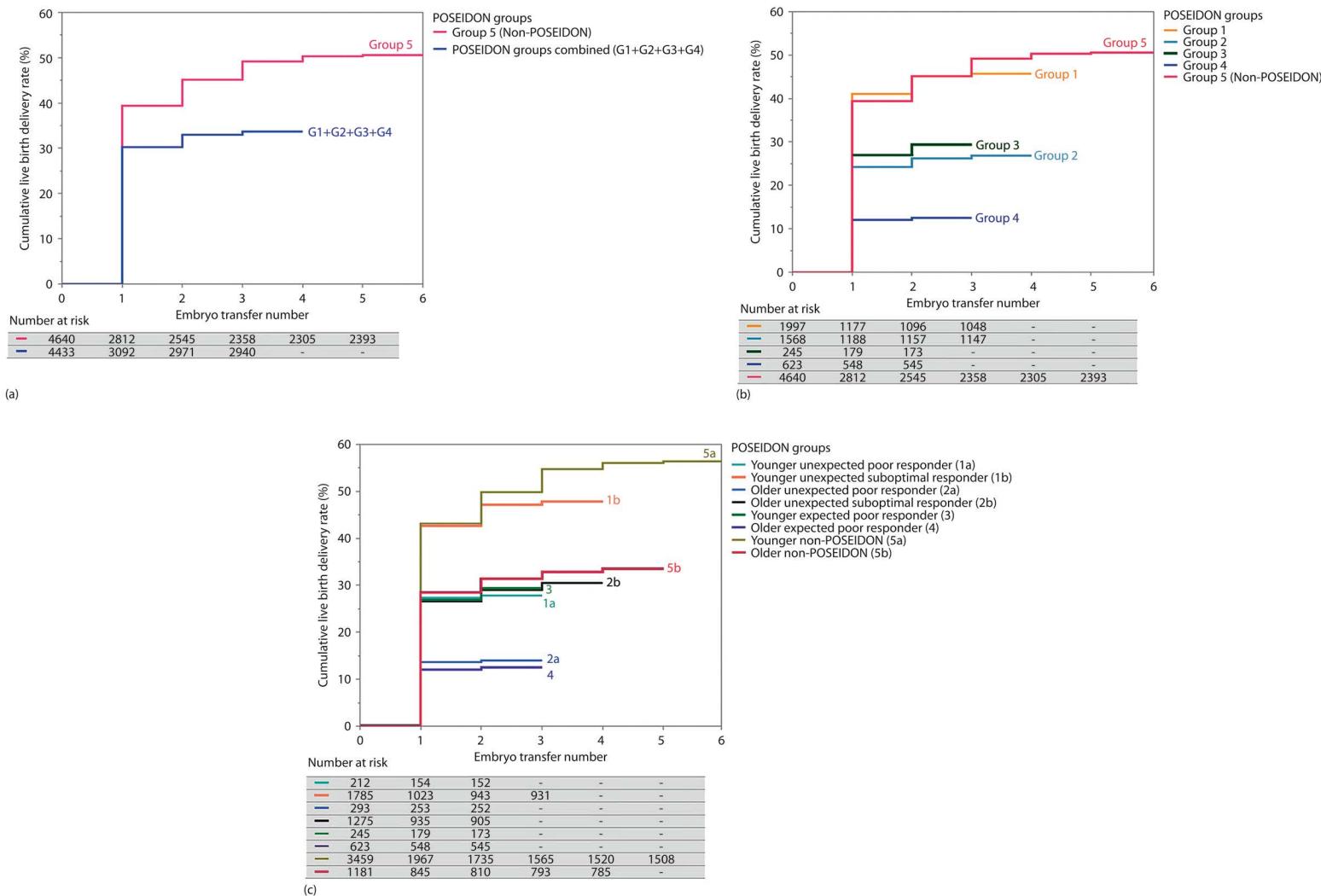


FIGURE 53.7 Survival plots for cumulative delivery in POSEIDON patients. Patients were stratified according to the POSEIDON criteria. Time-to-event plots were generated by (a) all POSEIDON patients—combined into a single group—and the control group of non-POSEIDON patients, (b) the four POSEIDON groups 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].)

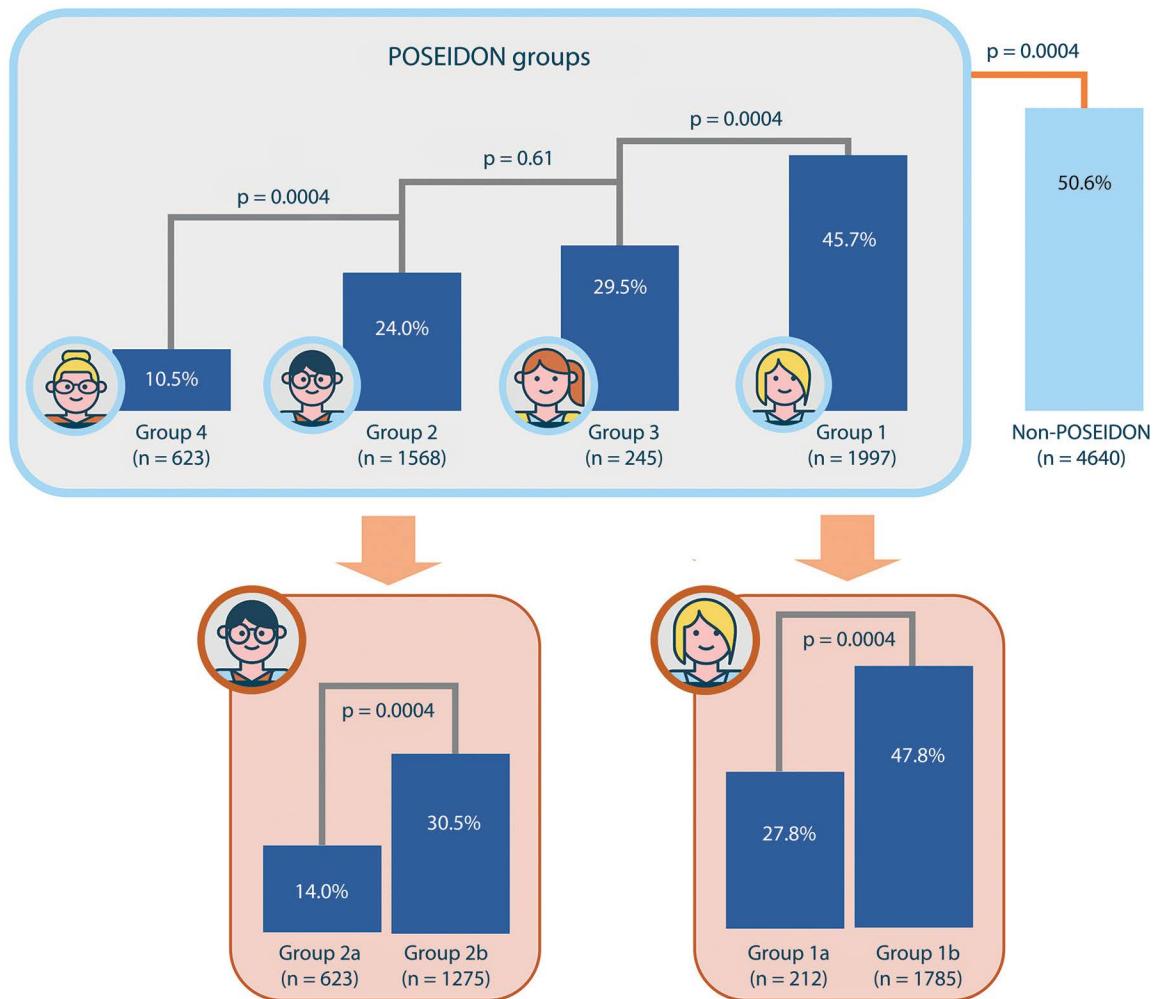


FIGURE 53.8 Cumulative delivery rates in POSEIDON patients. The prognosis of POSEIDON patients undergoing assisted reproductive technology, defined by the probability of achieving a delivery after the transfer of one or more embryos obtained from one aspiration IVF/ICSI cycle, is 50% lower than non-POSEIDON 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

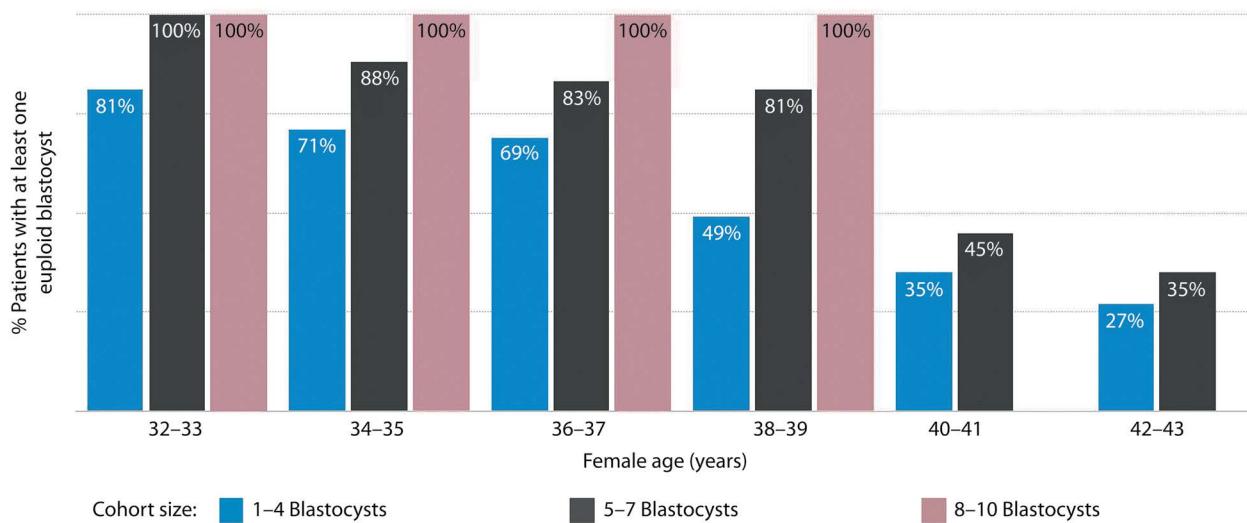


FIGURE 53.9 Frequency of patients with at least one euploid blastocyst stratified by age and embryo cohort size. Analysis of 1220 trophectoderm biopsies from 436 infertile couples undergoing ICSI and pre-implantation genetic testing for aneuploidy (PGT-A) by next-generation sequencing. The mean frequency 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

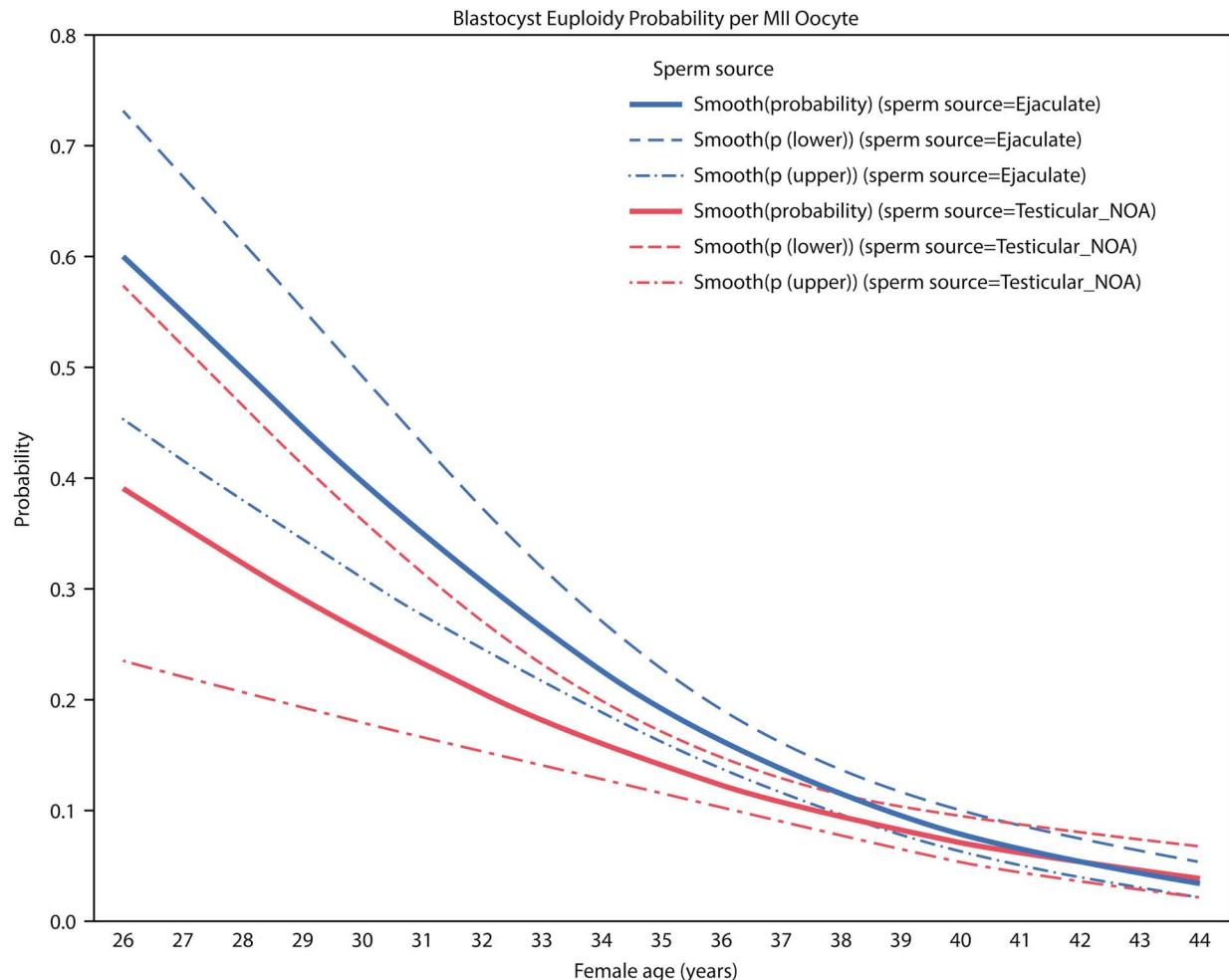


FIGURE 53.10 Blastocyst euploidy probability per metaphase II (MII) oocyte. The plots show the probability of a metaphase II (MII) oocyte turning into a euploid blastocyst as a function of female age. The estimated probabilities (solid curves) and their 95% confidence interval (dotted curves) are presented 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

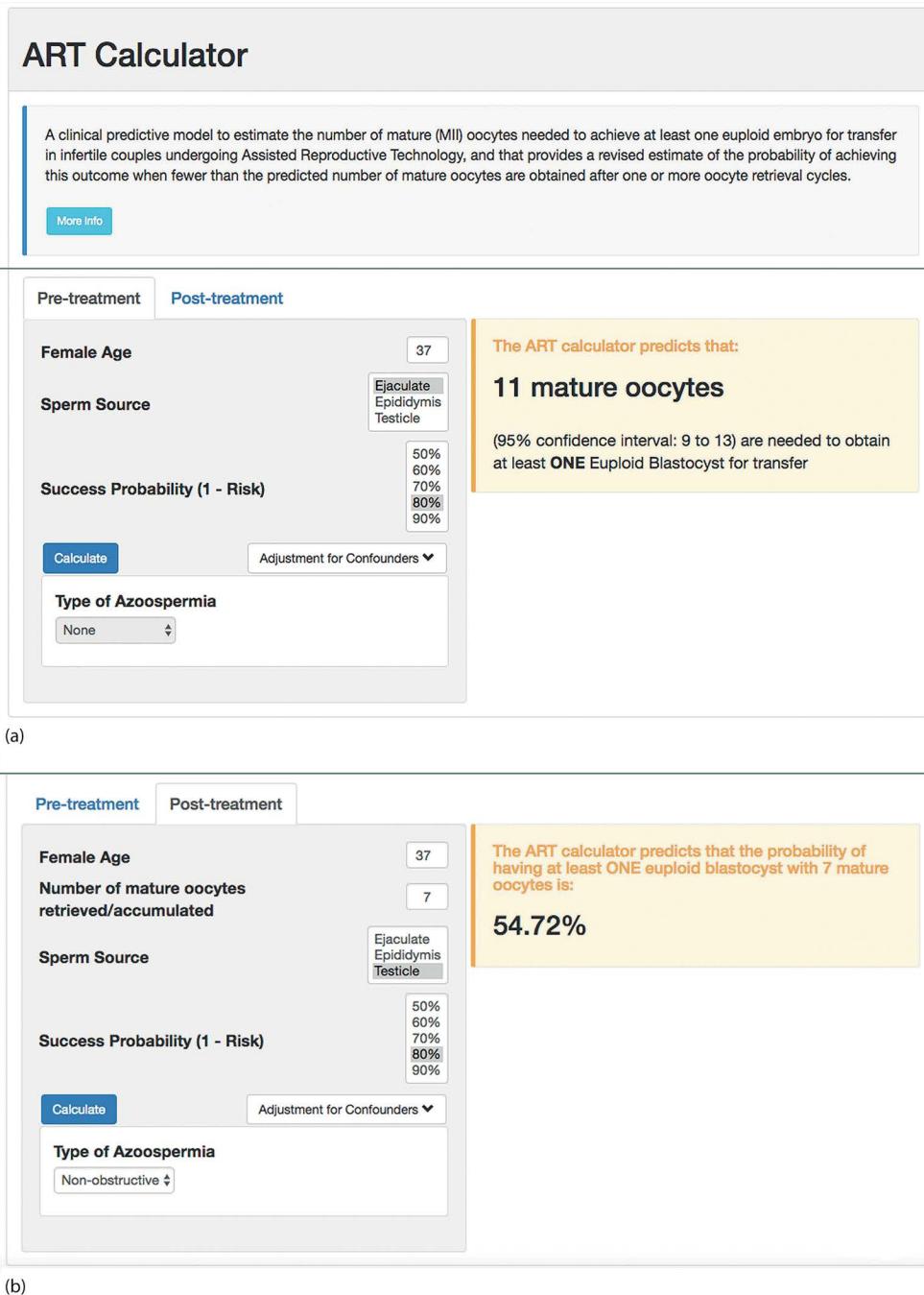


FIGURE 53.11 ART calculator. Online calculator to estimate the minimum number of mature oocytes required to obtain at least one euploid blastocyst for transfer in infertile patients undergoing IVF/ICSI cycles. The figure shows how the calculator can be used in an office-based setting. (a) Pre-treatment, 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].)

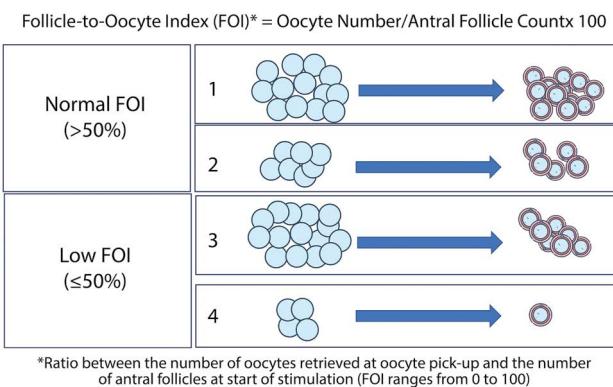


FIGURE 53.12 Follicle-to-oocyte index (FOI). Ovarian sensitivity to stimulation with exogenous gonadotropins as determined using the follicle-to-oocyte index (FOI). Case number 1 depicts a patient with normal FOI, in whom the number of oocytes retrieved was consistent with the AFC at the start of stimulation (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].

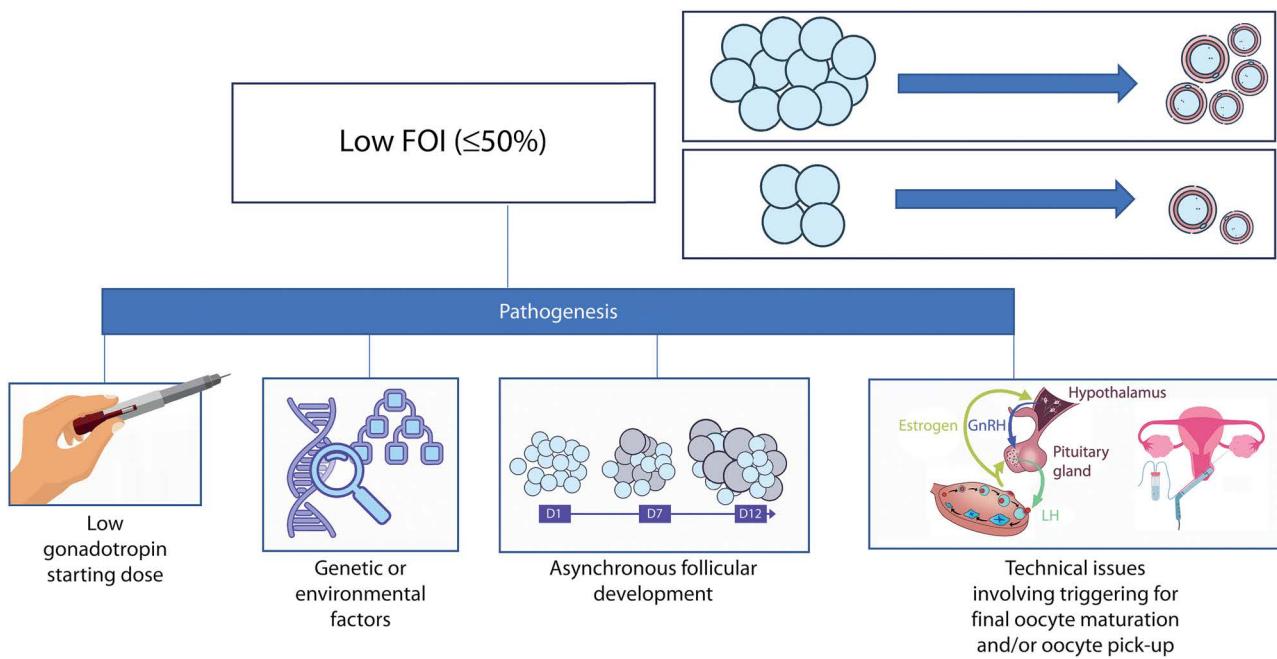


FIGURE 53.13 Possible causative factors for low follicle-to-oocyte indices. The figure depicts the main factors possibly associated with an inadequate FOI value. These factors are not mutually exclusive and include (i) low gonadotropin starting dose, (ii) ovarian hypo-response to gonadotropin stimulation 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

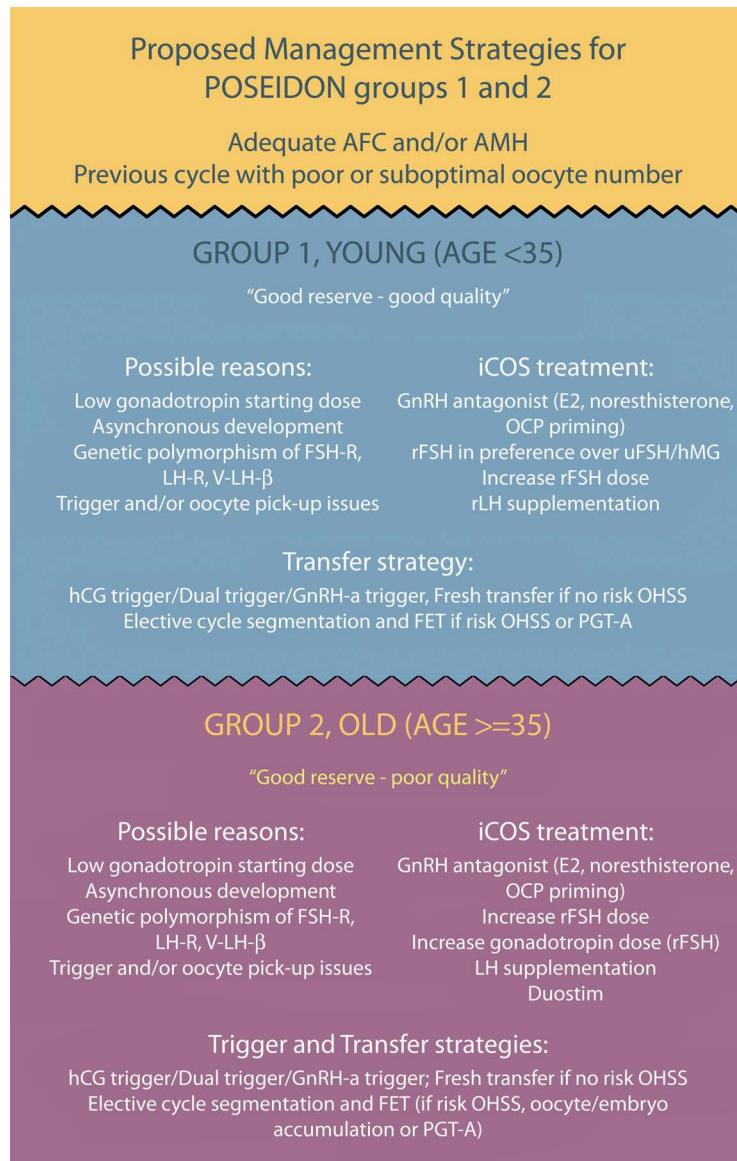


FIGURE 53.14 Proposed management strategies for POSEIDON groups 1 and 2 patients. Patients belonging to groups 1 and 2 share the same common feature of a poor (fewer than four) or suboptimal (four to nine) number of oocytes retrieved after a standard ovarian stimulation with exogenous gonadotropins despite 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].)

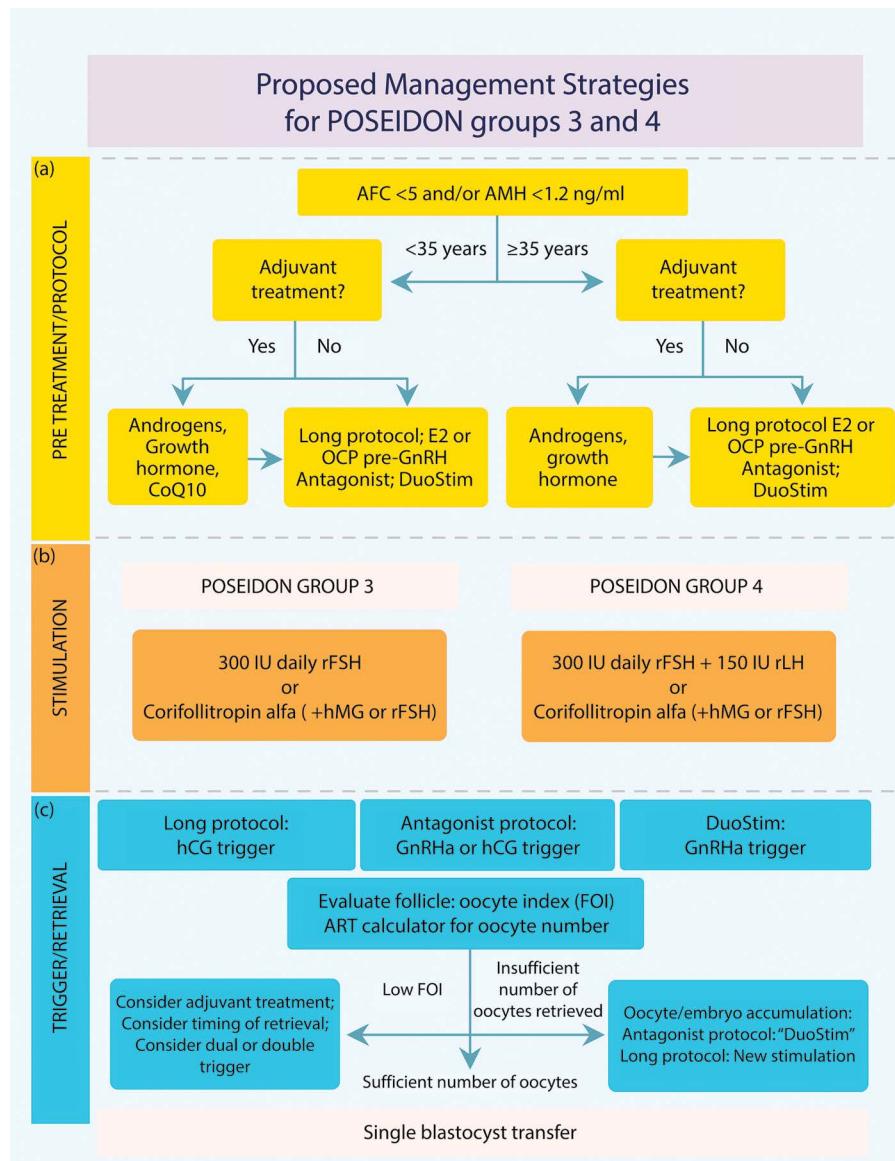


FIGURE 53.15 Proposed management strategies for POSEIDON groups 3 and 4 patients. Patients belonging to groups 3 and 4 share the same common feature of a poor ovarian reserve, which, independently of age, renders them at high risk of a poor reproductive ART outcome. These patients require special attention 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

- 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.
Results	<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.
Discussion	<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).

* 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.

Acknowledgements

The authors are grateful to Chloé Xilinas and Roberto Cavalieri from Med.E.A. for their help developing the figures. Furthermore, we are thankful to all POSEIDON group members for sharing their excitement in studying the low-prognosis patient and contributing their knowledge and ideas.

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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).

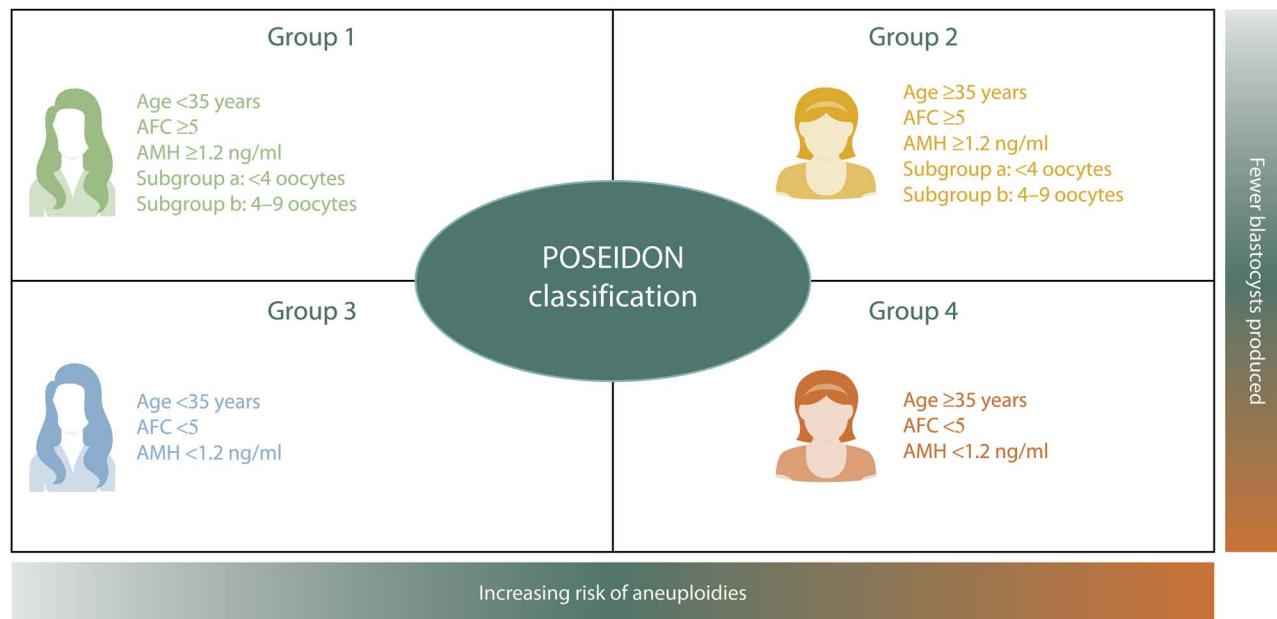


FIGURE 54.1 POSEIDON classification of poor prognosis patients. Four groups of poor-prognosis patients can be defined based on maternal age, ovarian reserve, previous response to controlled ovarian stimulation (COS), and expected aneuploidy rate at the blastocyst stage.

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

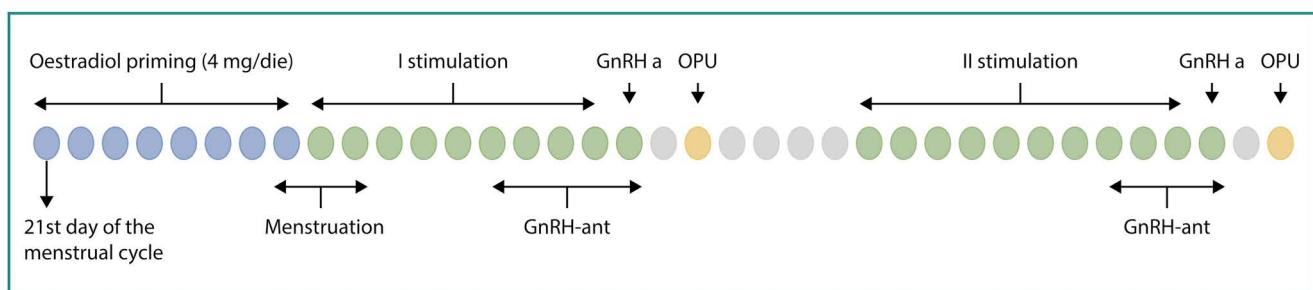


FIGURE 54.2 DuoStim protocol. Luteal oestradiol priming (4 mg/day of oestradiol valerate) is started on day 21 of the previous menstrual cycle to synchronize the follicular growth. After transvaginal ultrasound and basal ovarian assessment, on day 2 or 3 of the menstrual cycle, luteal oestradiol priming 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

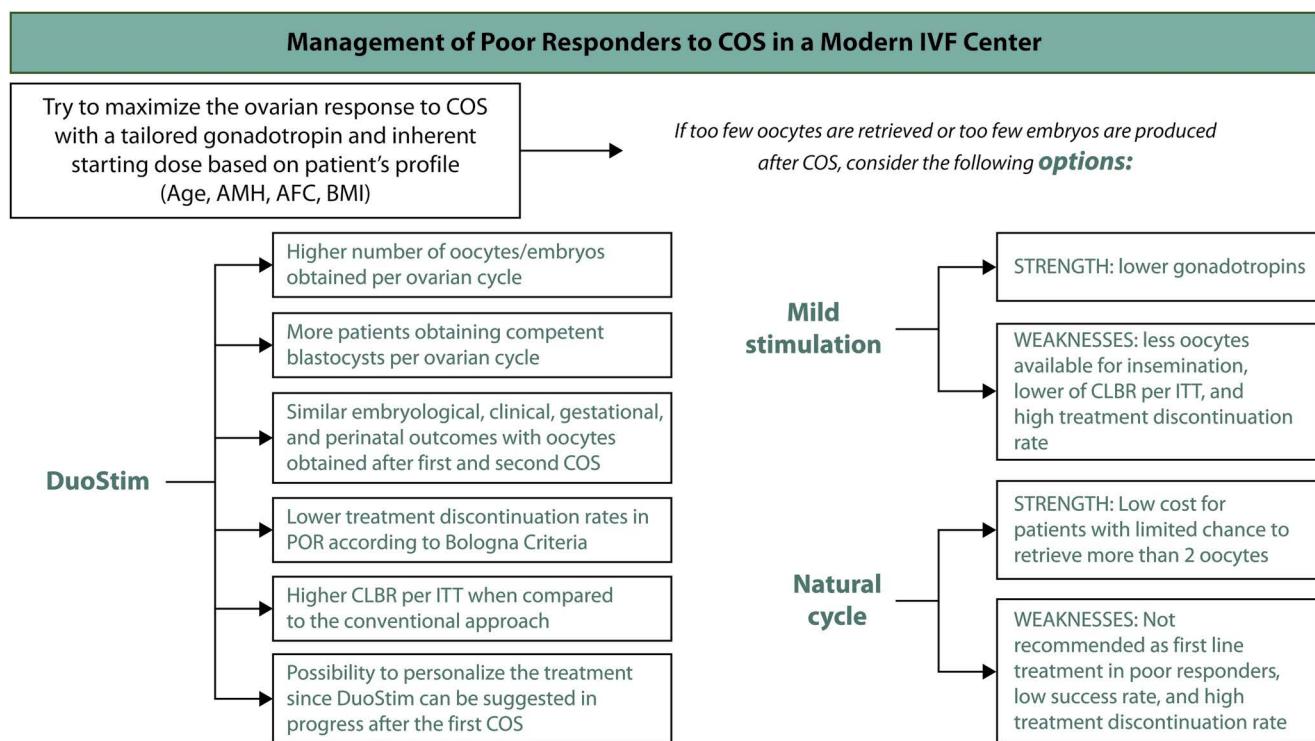


FIGURE 54.3 Summary of the main approaches to manage poor prognosis patients in a modern IVF centre. The first line approach is to adopt a tailored controlled ovarian stimulation (COS) strategy to attempt at maximizing the ovarian response, based on maternal age, AMH, antral follicle count (AFC), and 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.

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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

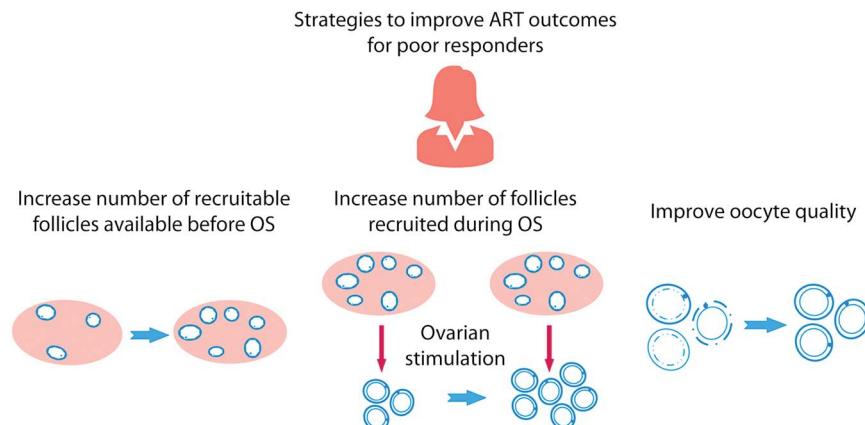


FIGURE 55.1 Strategies to treat poor ovarian response after ovarian stimulation for ART.

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).

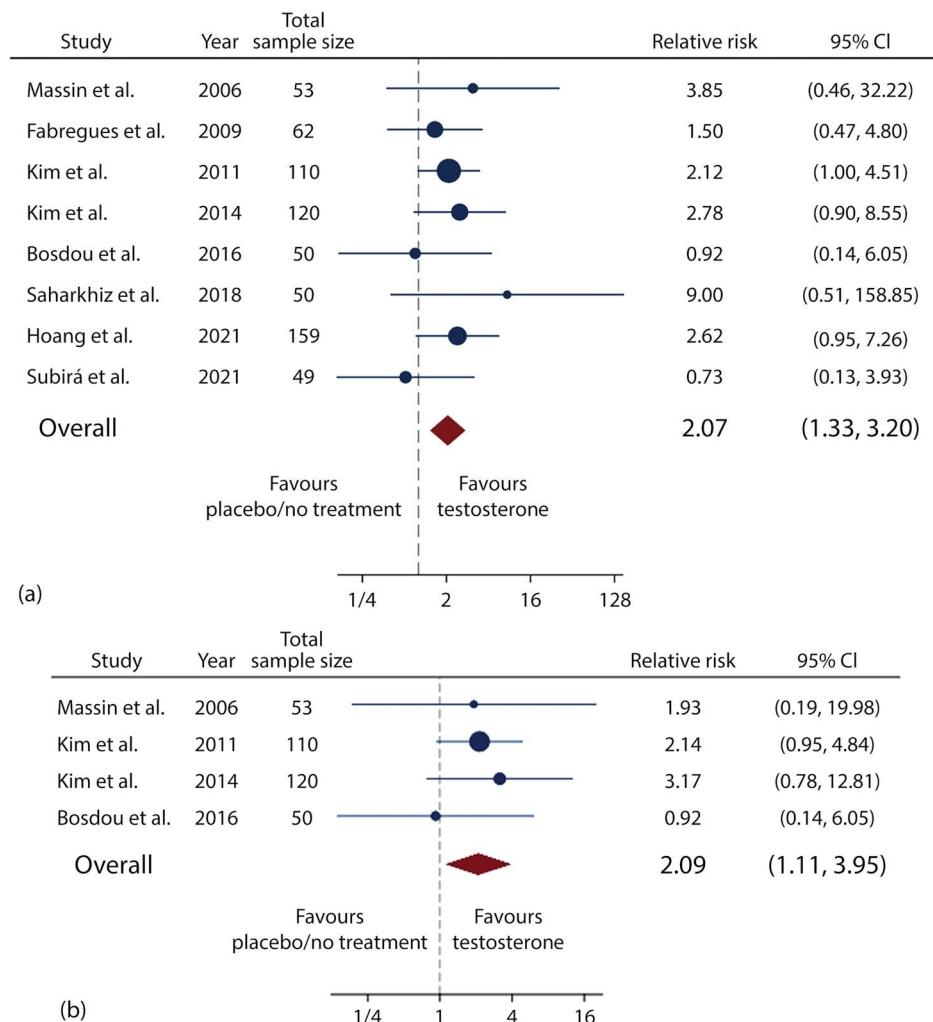


FIGURE 55.2 Statistical synthesis of studies evaluating the use of testosterone pre-treatment in poor responders based on Neves et al. (2022) [52]. (a) Clinical pregnancy. (b) Live birth.

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

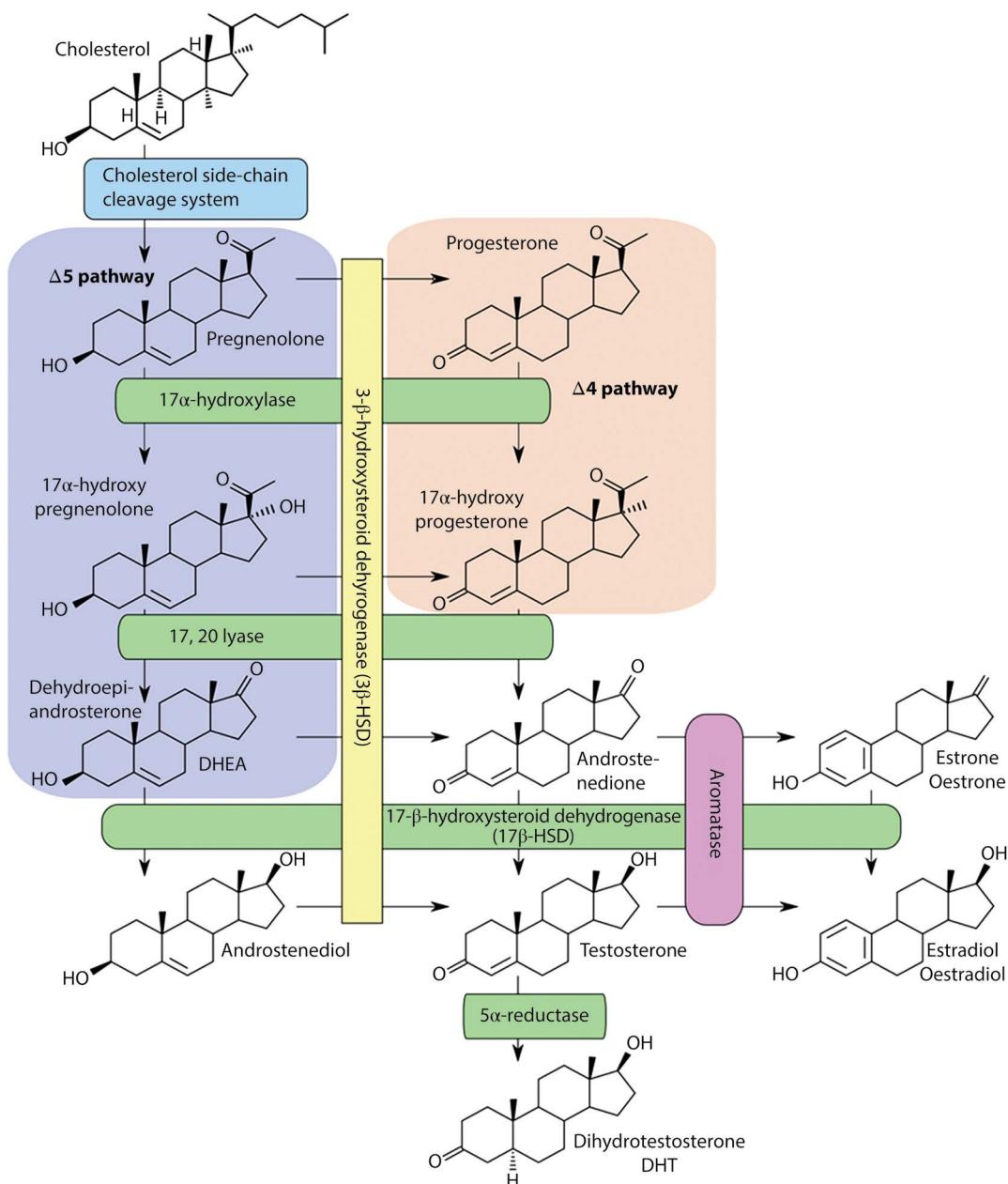


FIGURE 55.3 Steroidogenesis of androgens and oestrogen.

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

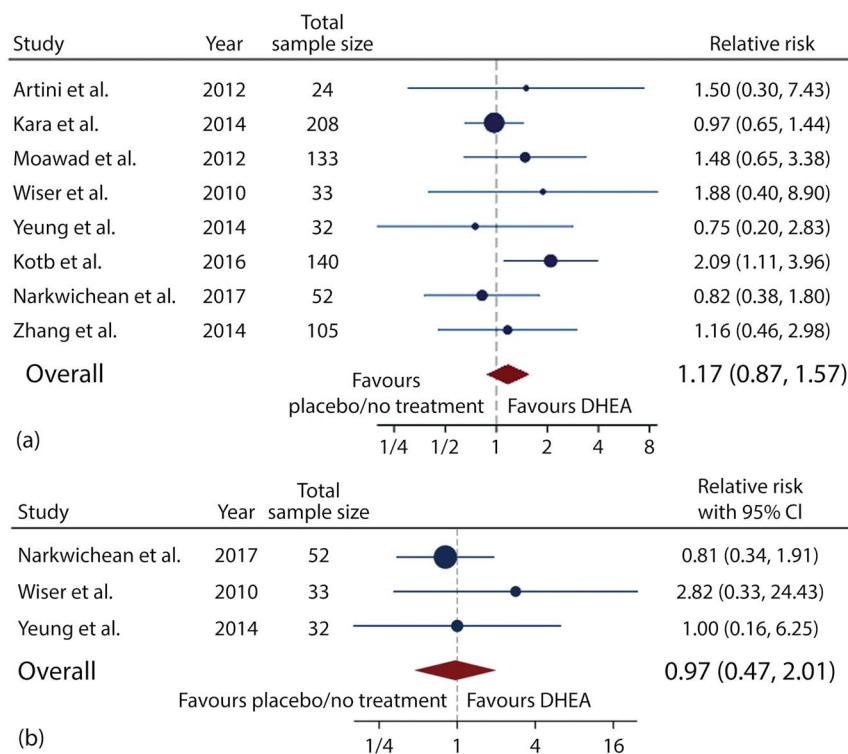


FIGURE 55.4 Statistical synthesis of studies evaluating the use of dehydroepiandrosterone (DHEA) pre-treatment in poor responders based on Neves et al. (2022) [52]. (a) Clinical pregnancy. (b) Live birth.

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

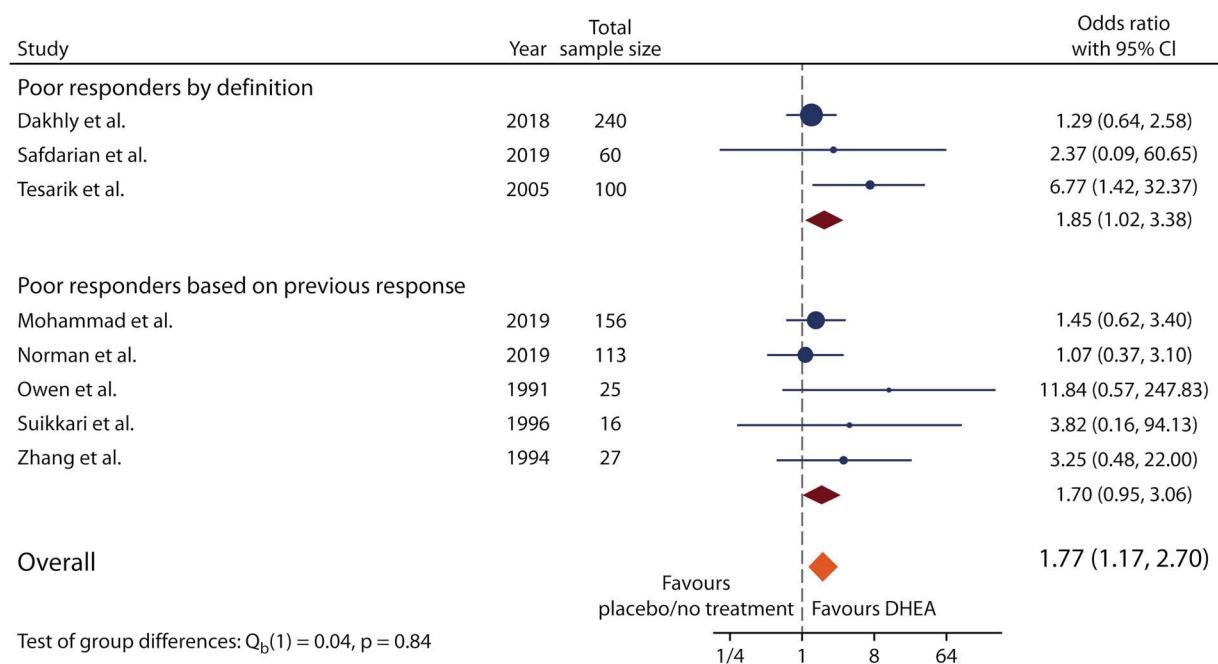


FIGURE 55.5 Statistical synthesis of studies evaluating the use of human growth hormone (hGH) pre-treatment in poor responders based on Sood et al. (2021) [90].

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.

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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).

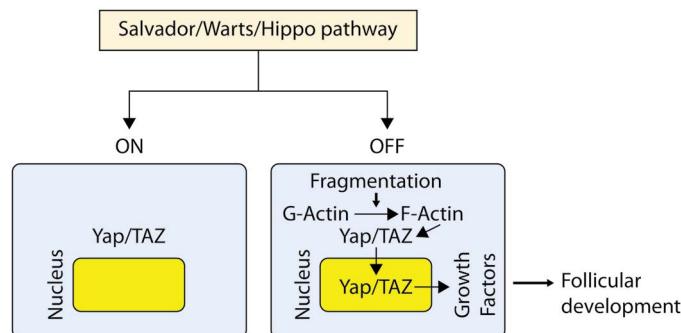


FIGURE 56.1 The Hippo pathway.

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

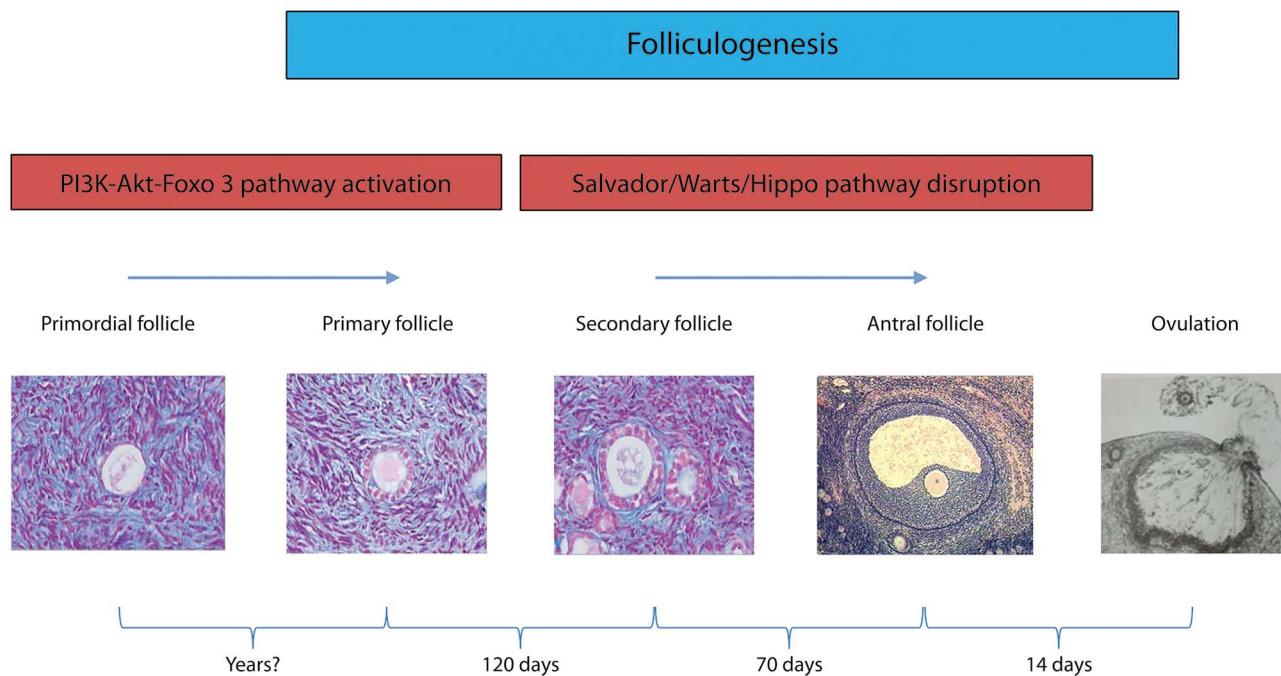


FIGURE 56.2 Folliculogenesis.

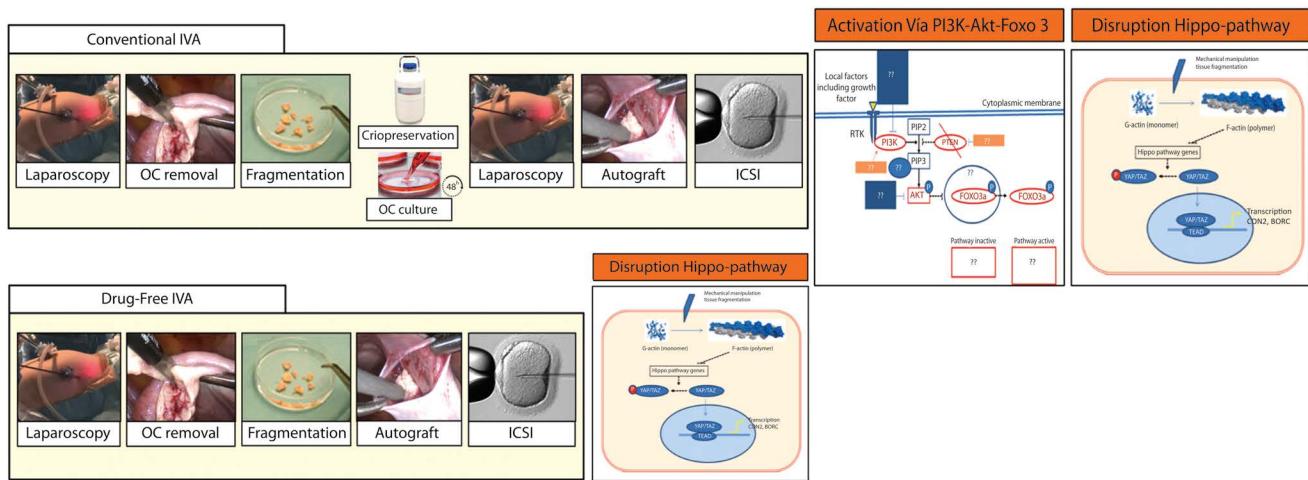


FIGURE 56.3 Conventional and drug-free IVA. (From [29] with permission.)

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.

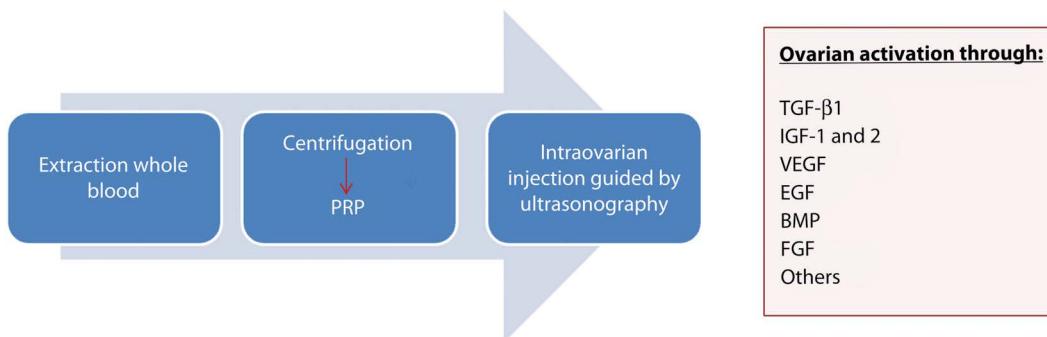


FIGURE 56.4 Ovarian activation via the PRP technique.

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

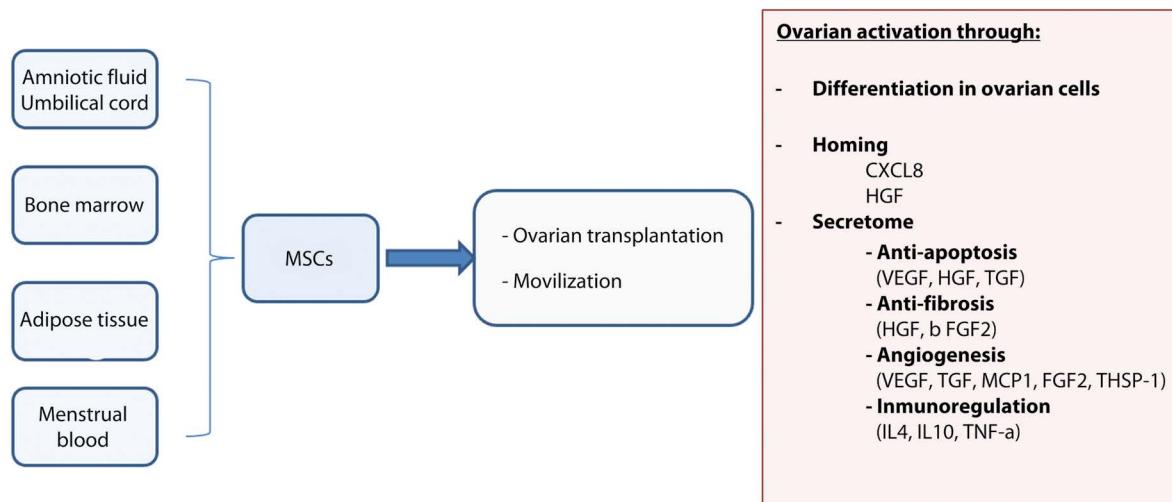


FIGURE 56.5 Ovarian activation via the secretome.

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].

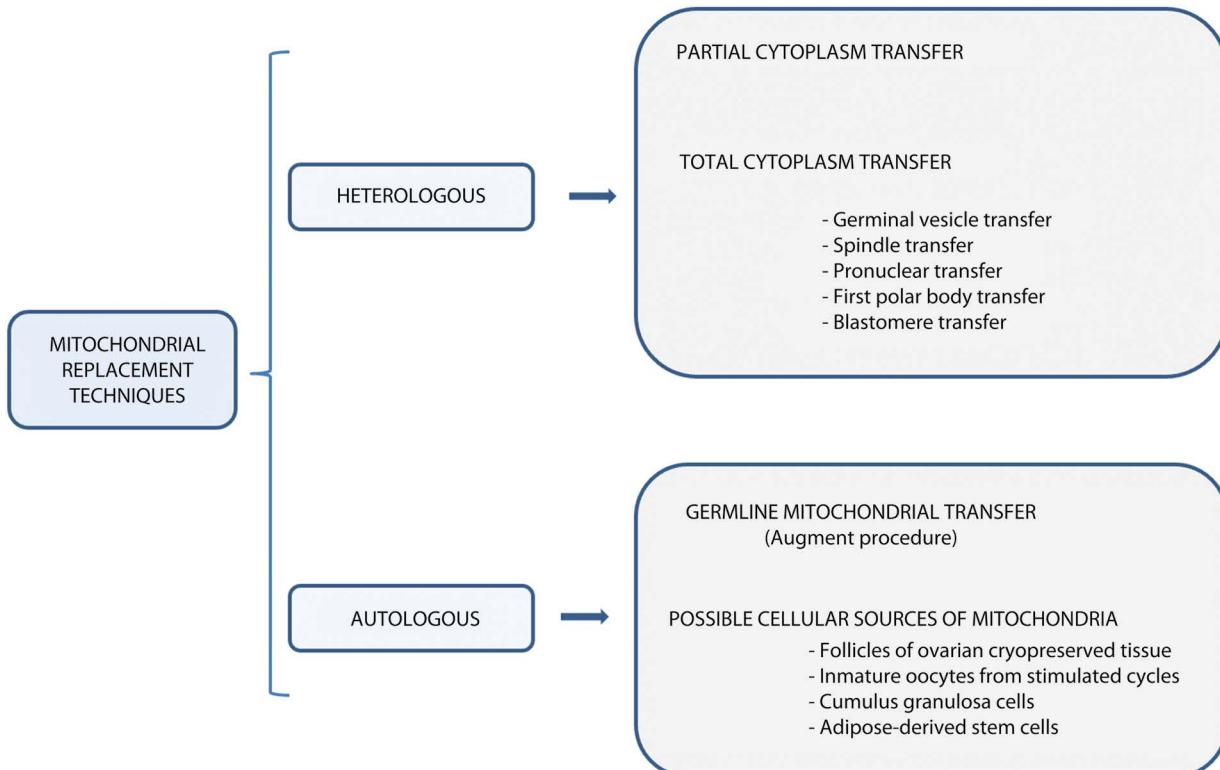
Partial cytoplasm transfer consists of the aspiration of cytoplasm from a donor oocyte and its introduction into the patient's

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.

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REPEATED IMPLANTATION FAILURE

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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.

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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.

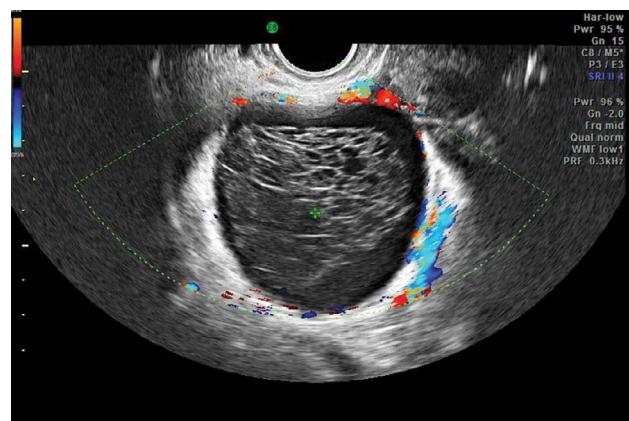


FIGURE 58.1 Haemorrhagic cyst with no flow into the cyst.

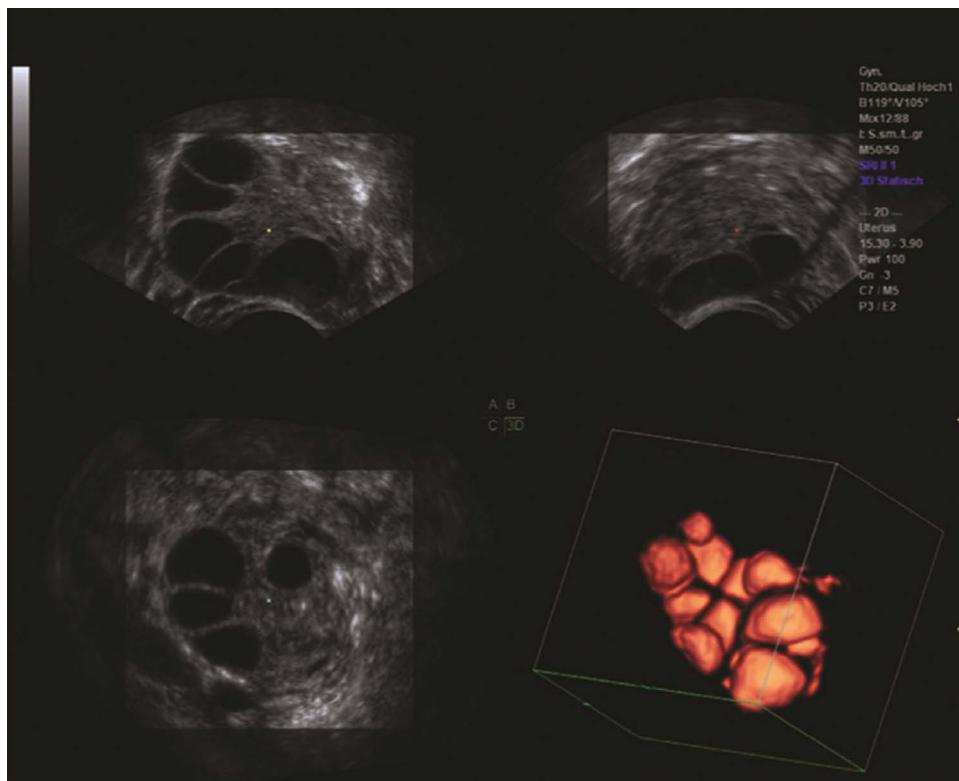


FIGURE 58.2 3D antral follicle count using inverse mode.

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

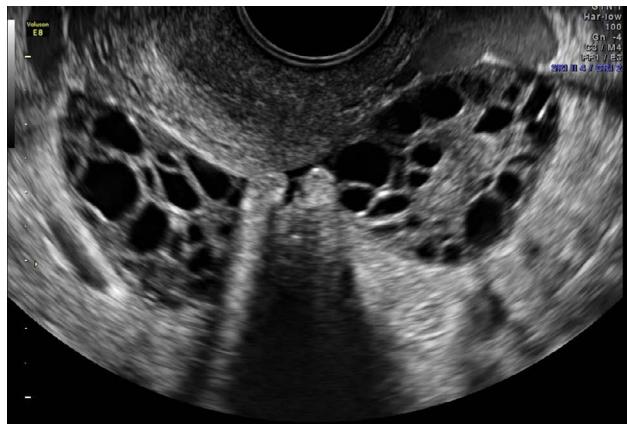


FIGURE 58.5 Polycystic-appearing ovary.

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

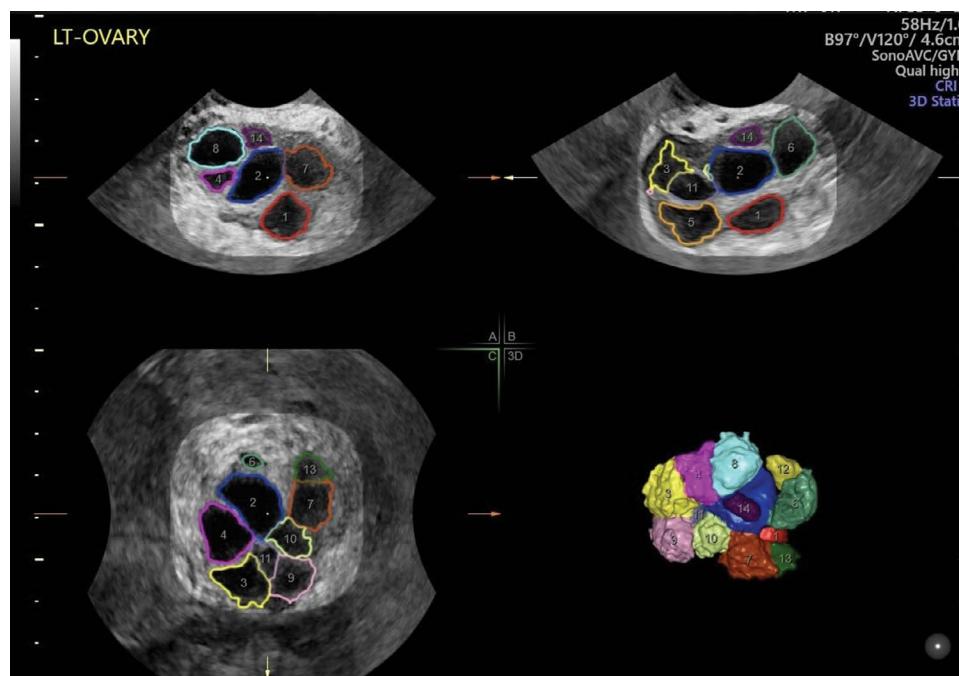


FIGURE 58.6 Automated follicular monitoring with rendered view.

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

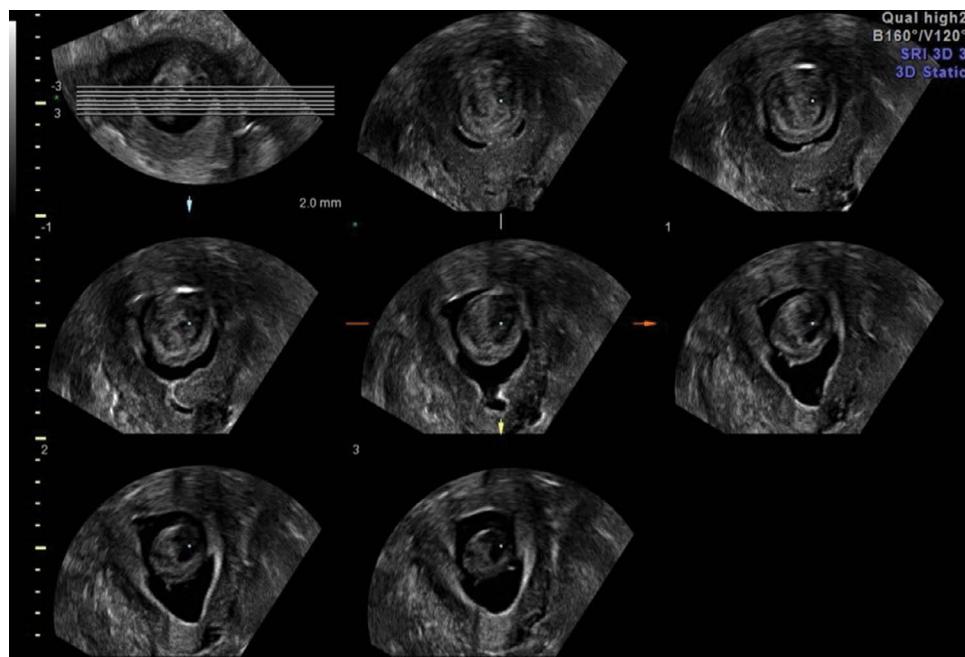


FIGURE 58.8 Saline sonogram of submucosal fibroid multiplanar view.

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

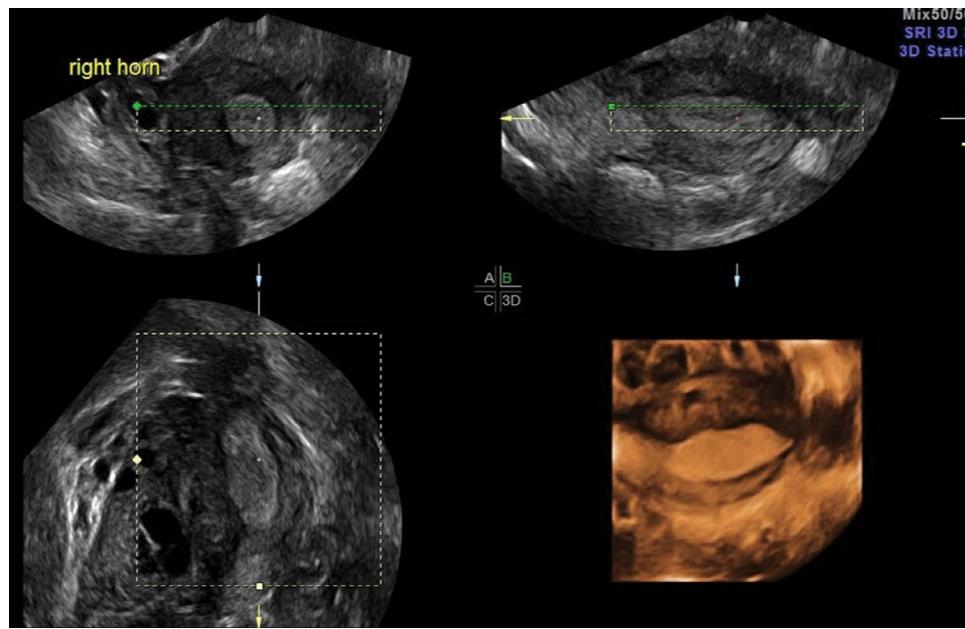


FIGURE 58.11 3D unicornuate uterus manipulation.

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

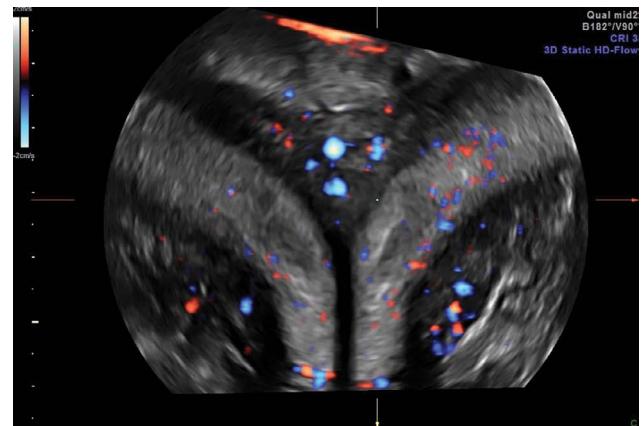


FIGURE 58.13 3D of complete uterine septum.

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

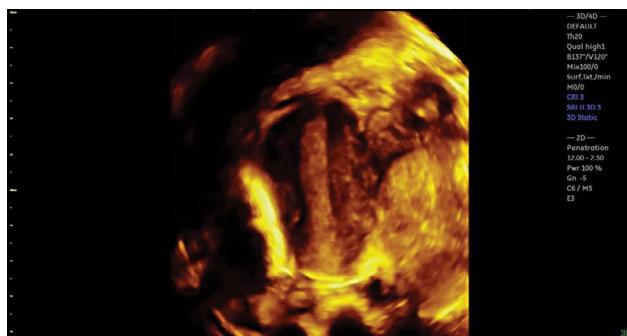


FIGURE 58.12 Unicornuate uterus with noncommunicating horn.

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

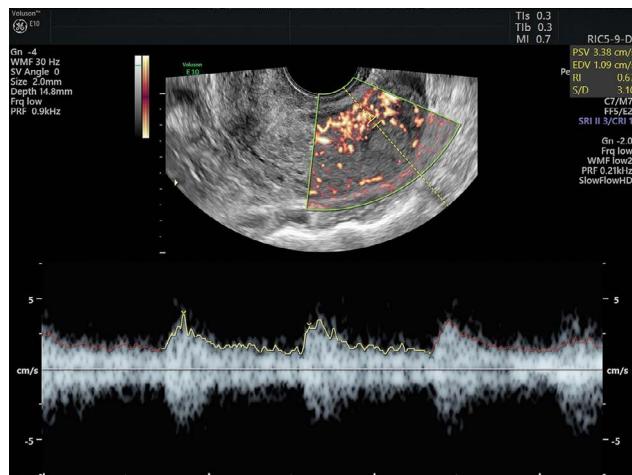


FIGURE 58.14 Slowflow Doppler of the endometrium.

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.

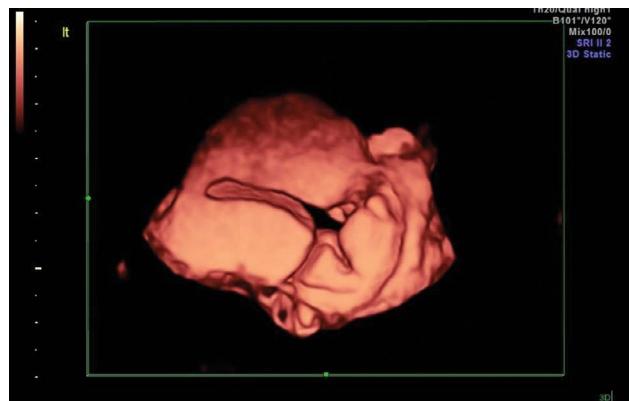


FIGURE 58.16 3D rendered view of hydrosalpinx.

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

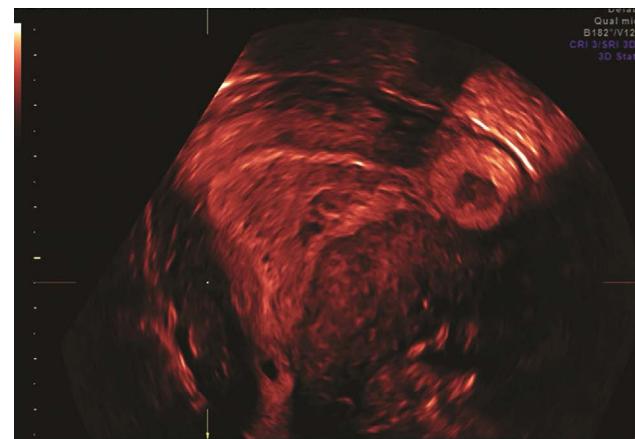


FIGURE 58.17 Ectopic pregnancy coronal view.

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.

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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

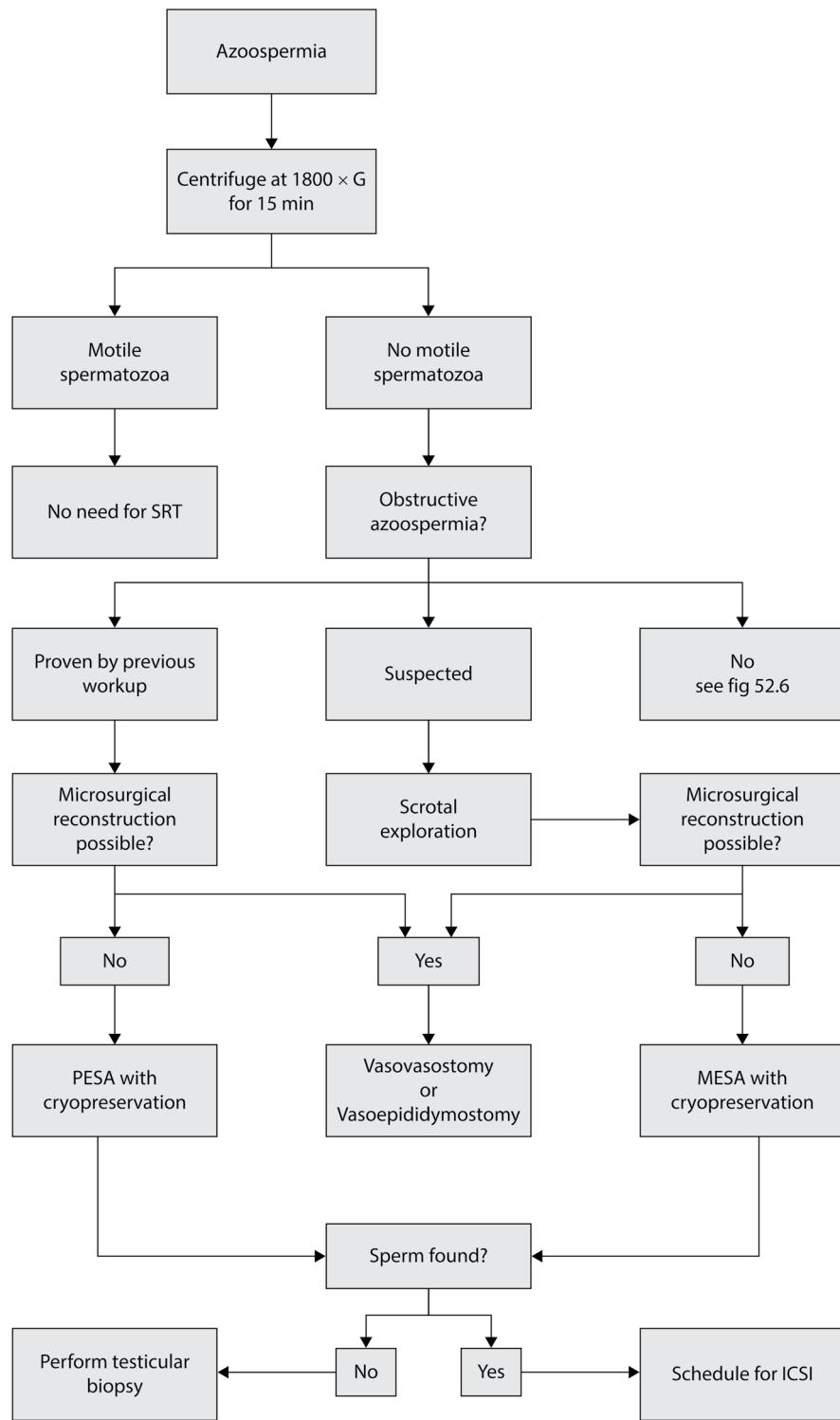


FIGURE 59.3 Treatment algorithm for patients with obstructive azoospermia. Abbreviations: ICSI, intracytoplasmic sperm injection; MESA, micro-surgical epididymal sperm aspiration; PESA, percutaneous epididymal sperm aspiration; SRT, sperm recovery technique.

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.

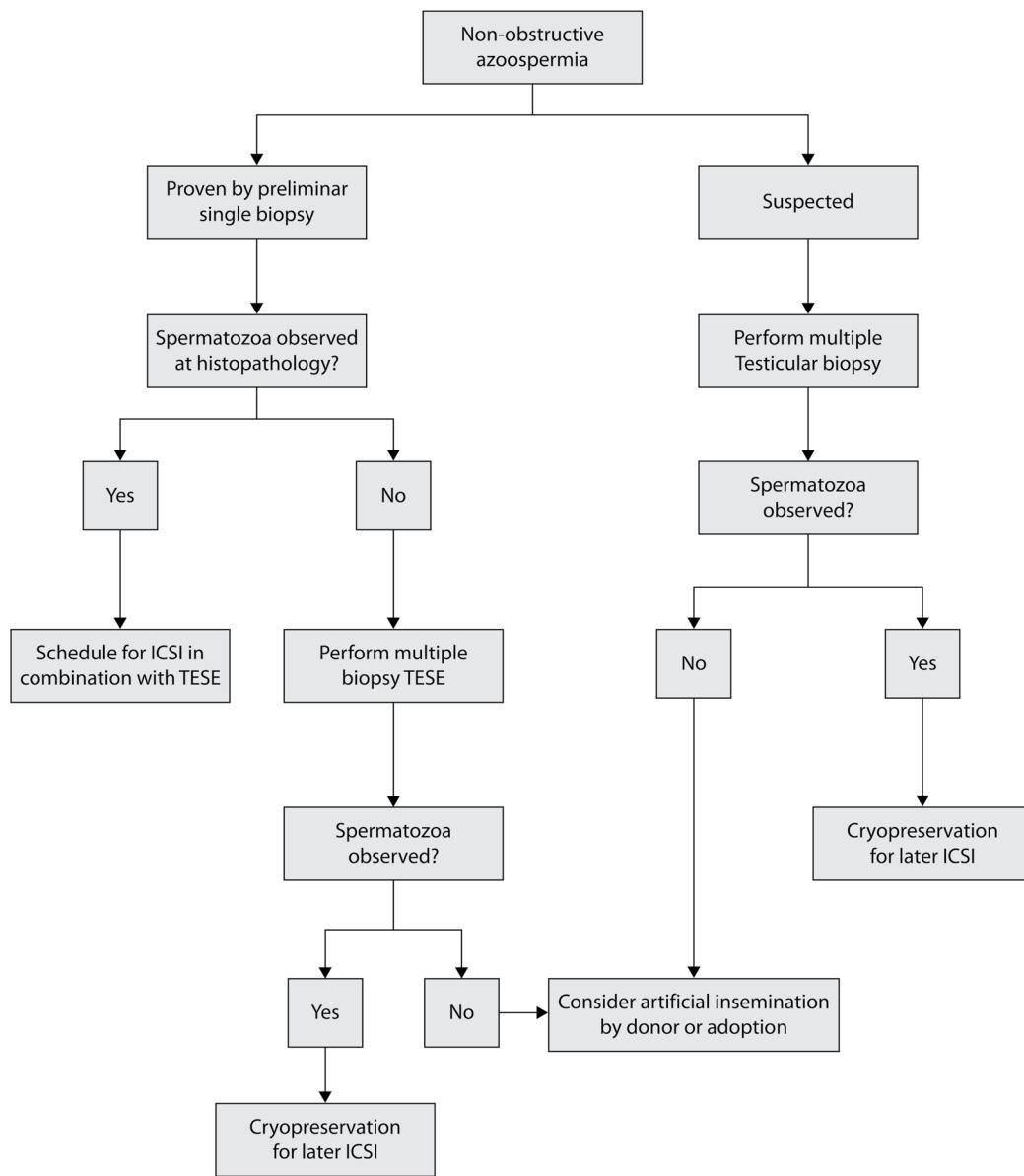


FIGURE 59.7 Treatment algorithm for patients with non-obstructive azoospermia. Abbreviations: ICSI, intracytoplasmic sperm injection; TESE, testicular sperm extraction.

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

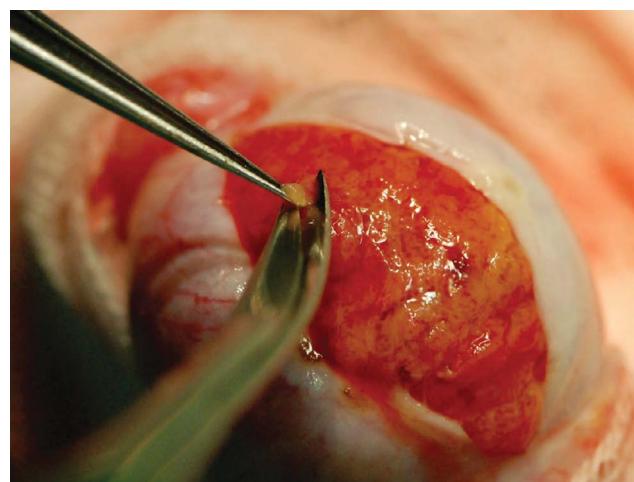


FIGURE 59.9 Testicular sampling by microscope-guided testicular sperm extraction (TESE) or micro-TESE. Under magnification, a dilated seminiferous tubule is excised using microscissors.

(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 orchietomy [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]

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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.

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CYCLE REGIMES FOR FROZEN-THAWED EMBRYO TRANSFER

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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.

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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

follicles 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.

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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)			Number of Cycles	Clinical Pregnancies (% Cycle)
				Study	Group		
Mahadevan [83]	I–IV	14	14	Inoue [74]	I	111	40
	Tubal	261	10		II	78	42
Wardle [84]	I–IV	17	6	Olivennes [76]	III	51	47
	Tubal	47	11		IV	69	42
Chillik [61]	I/II	10	60	Geber [73]	Other	372	44
	III/IV	14	7		I–IV	360	29
Matson [63]	I	24	13	Dmowski [71]	Tubal	160	36
	II	37	14		I/II	100	29
	III	36	6		III/IV	29	52
	VI	57	2		Tubal	1139	41
	Tubal	40	18		I/II	89	25
Sharma [85]	I/II	135	16	Arici [86]	III/IV	30	30
	III/IV	141	8		Other	118	21
	Tubal	994	13		I/II	43	12
Oehninger [62]	I/II	191	24	Bergendal [72]	III/IV	46	15
	III/IV	35	20		Tubal	147	24
Yovich [64]	I/II	61	13	Pal [75]	I–IV	65	28
	III/IV	93	3		Tubal	98	30
	Tubal	49	14		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.

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POLYCYSTIC OVARY SYNDROME AND ASSISTED REPRODUCTION

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Introduction

Polyzystic 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 *polycystic 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.

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FERTILITY PRESERVATION STRATEGIES

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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.

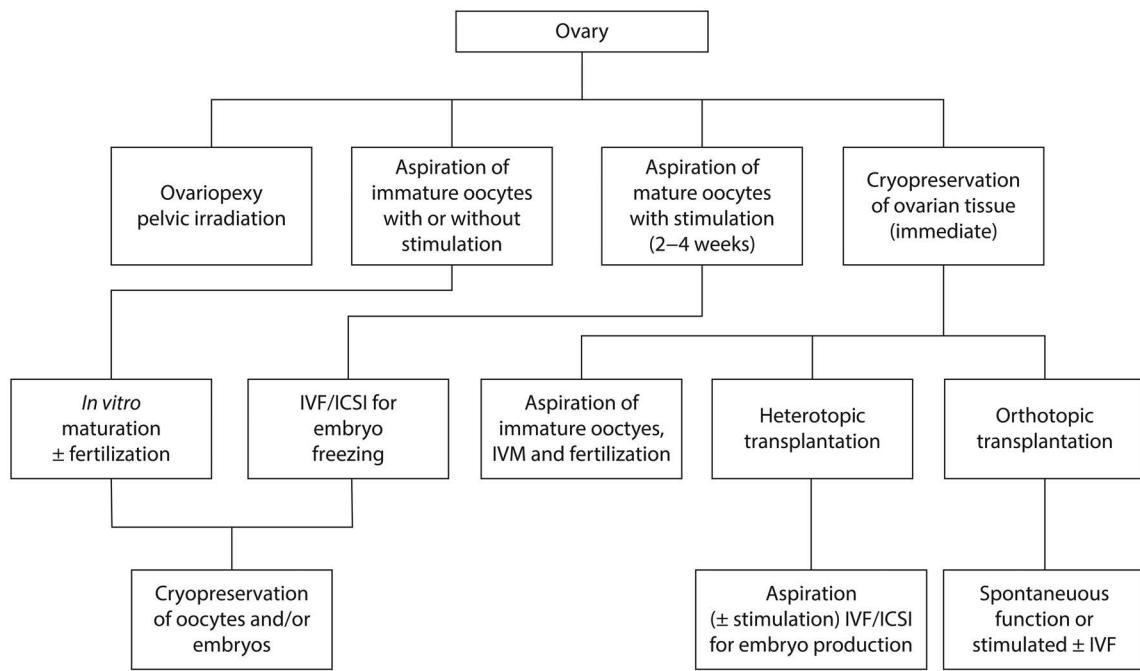


FIGURE 65.1 Options for fertility preservation in women. Abbreviations: IVM, *in vitro* maturation; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

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

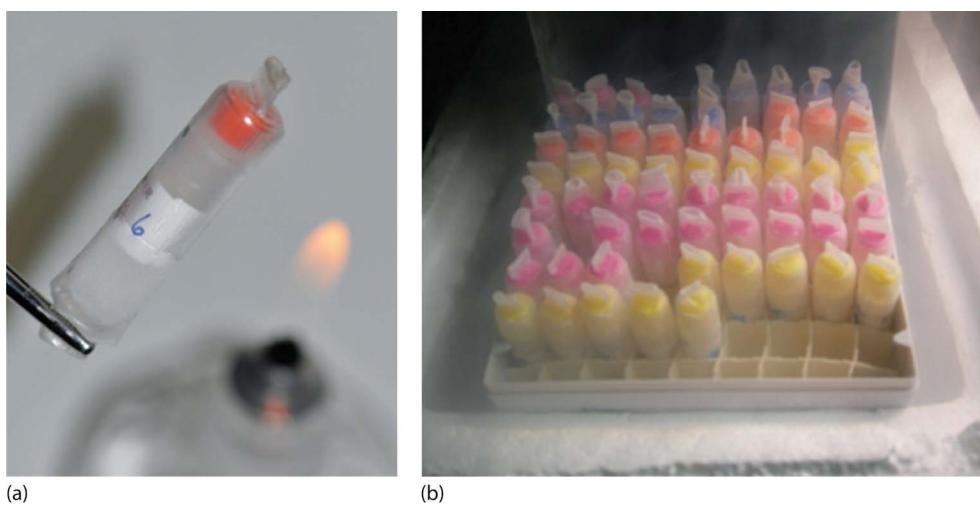


FIGURE 65.4 Dobbelt sealing and storage of cryo-tubes. (a) Once frozen, the cryo-tubes containing the ovarian tissue are double sealed in CryoFlex, (b) before long-term storage. Notice the different colour codes for each patient.

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,

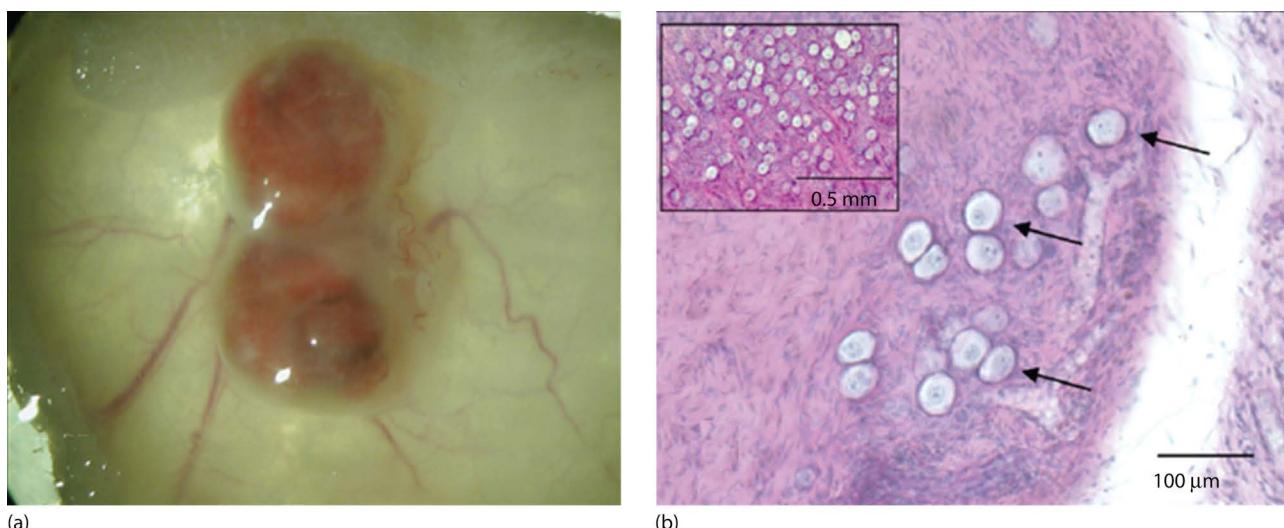


FIGURE 65.5 (a) Two pieces of frozen-thawed human ovarian cortex (5×5 mm) transplanted under the skin of an oophorectomized mouse. After two weeks, macroscopically visible revascularization was established. (b) Human ovarian cortex kept on ice for 20 hours prior to freezing. Thawed tissue was transplanted 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

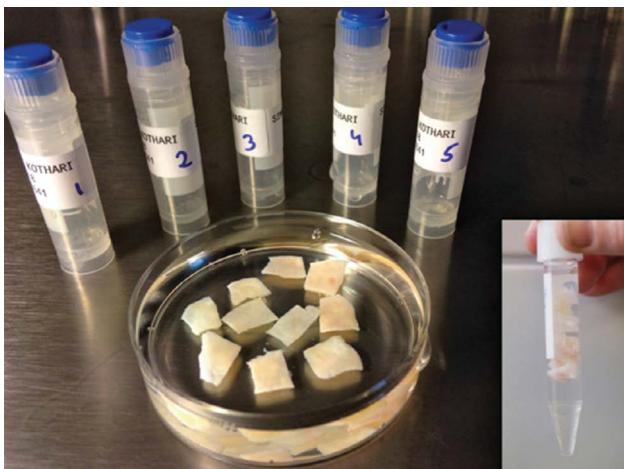


FIGURE 65.6 Three-step thawing procedure. Pieces of cortex are transferred to thawing solutions with decreasing concentrations of cryo-protectant. The inset shows the final step of thawing, and the tissue is subsequently brought to the operating theatre for transplantation.

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

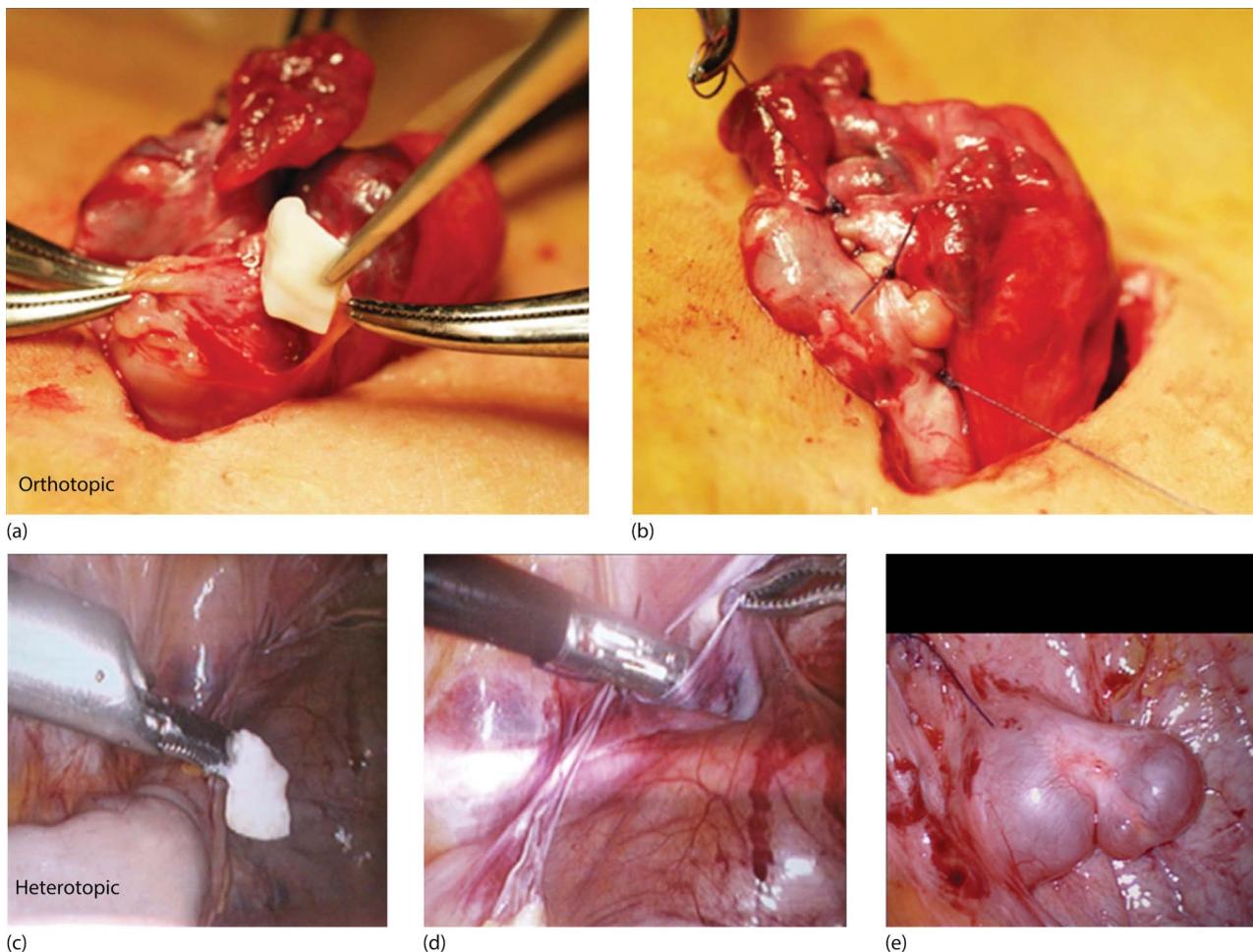


FIGURE 65.7 Transplantation of cryopreserved ovarian tissue. (a and b) Orthotopic transplantation: pieces of thawed ovarian cortex being transplanted in a subcortical pocket in the *in situ* ovary. (c and d) Heterotopic transplantation: pieces of thawed ovarian cortex being transplanted in a subperitoneal pocket corresponding to the pelvic wall. (e) Two human antral follicles at a heterotopic graft site several years after transplantation of thawed ovarian tissue.

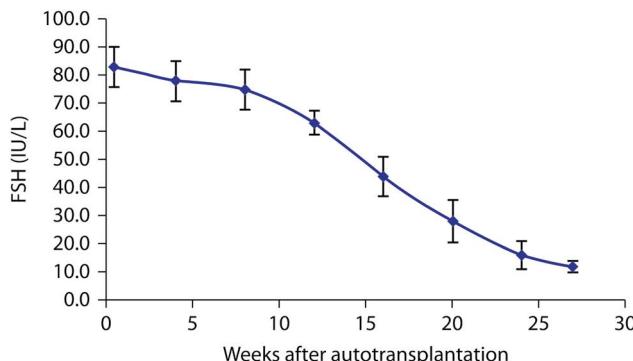


FIGURE 65.8 Restoration of ovarian function serum levels of FSH (IU/L) in 12 Danish patients after autotransplantation of frozen-thawed ovarian tissue (mean \pm standard error of the mean). Abbreviation: FSH, follicle-stimulating hormone.

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].

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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.

Support and sponsorship

This work was supported by grants from Jane and Dan Olsson Foundation for Science, the Swedish Research Council, and the Knut and Alice Wallenberg Foundation.

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	-
DD (Czech Rep)	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.

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VIRAL DISEASE AND ART

Carole Gilling-Smith

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)

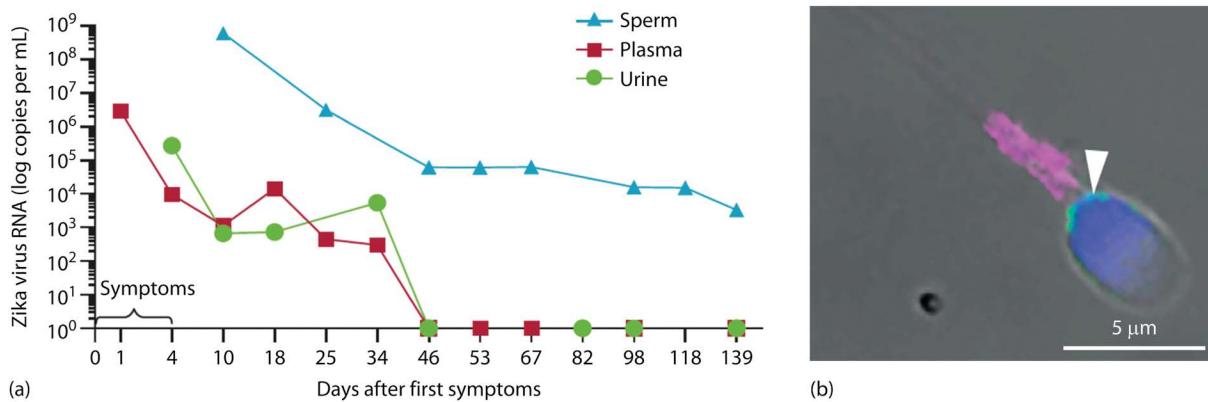


FIGURE 67.1 Zika virus infects spermatozoa. (a) The kinetics of Zika virus RNA detection in plasma, urine, and semen quantified by real-time polymerase chain reaction (RealStar Zika Virus RT-PCR Kit 1.0, Altona Diagnostics GmbH, Hamburg, Germany). (b) Immunohistochemical detection of Zika virus (green; 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.

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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 amenorrheic 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 luteum 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

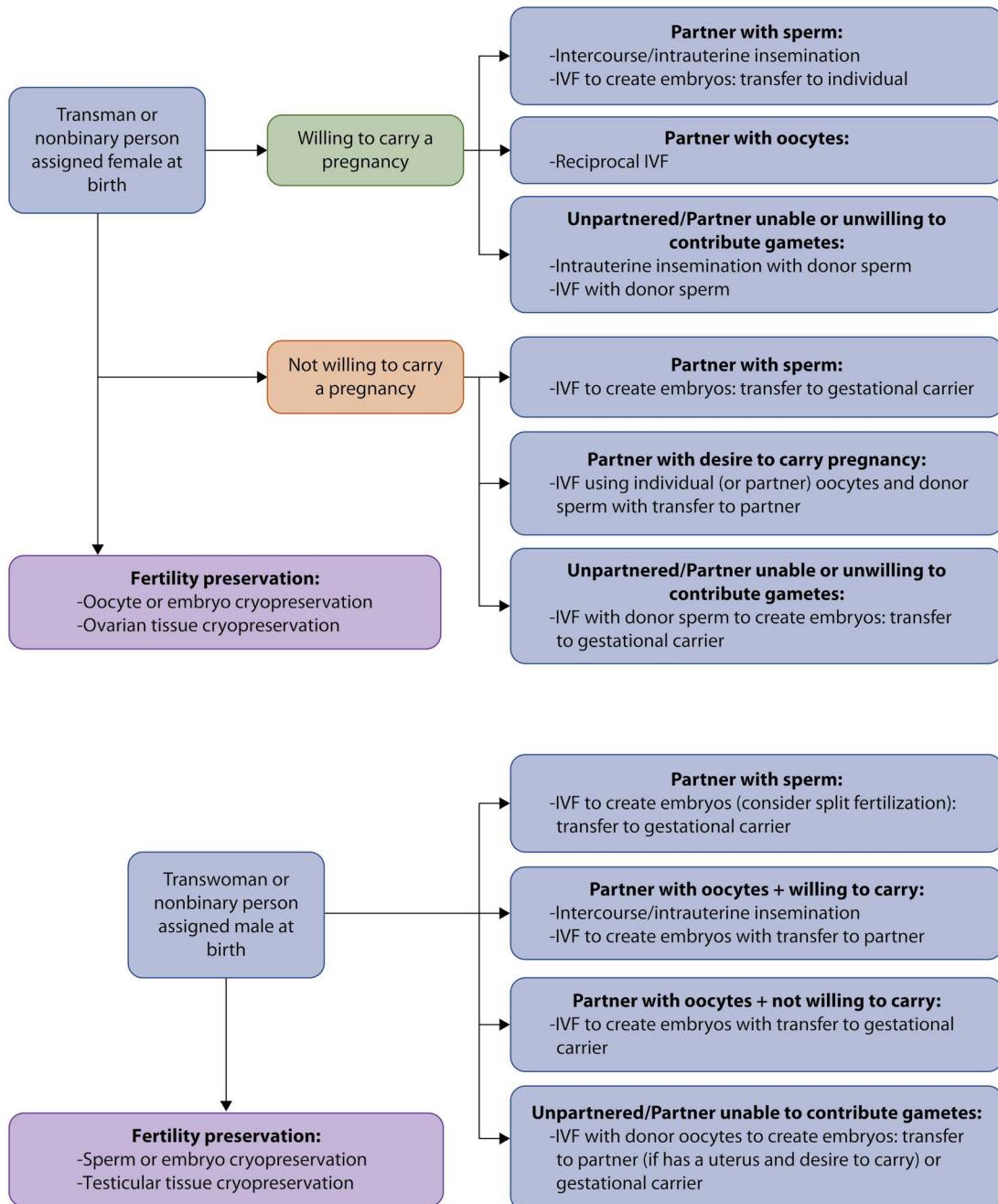


FIGURE 68.1 Reproductive options for transgender and nonbinary individuals.

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.

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OVARIAN HYPERSTIMULATION SYNDROME

Federica Di Guardo and Christophe Blockeel

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.

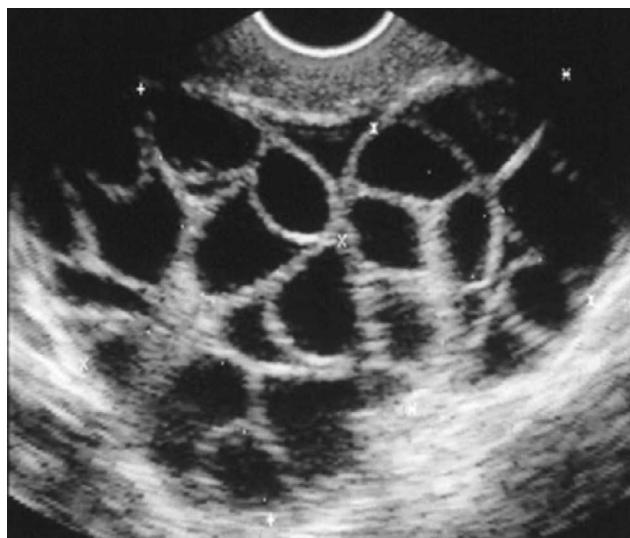


FIGURE 69.1 Hyperstimulated ovaries following ovarian stimulation. (Image adapted from Smith, Laura P. “Ultrasound and ovarian hyperstimulation syndrome.” *Ultrasound Imaging in Reproductive Medicine*. Springer, Cham, 2019. 321–333.)

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.

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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 F2 α , 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.

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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

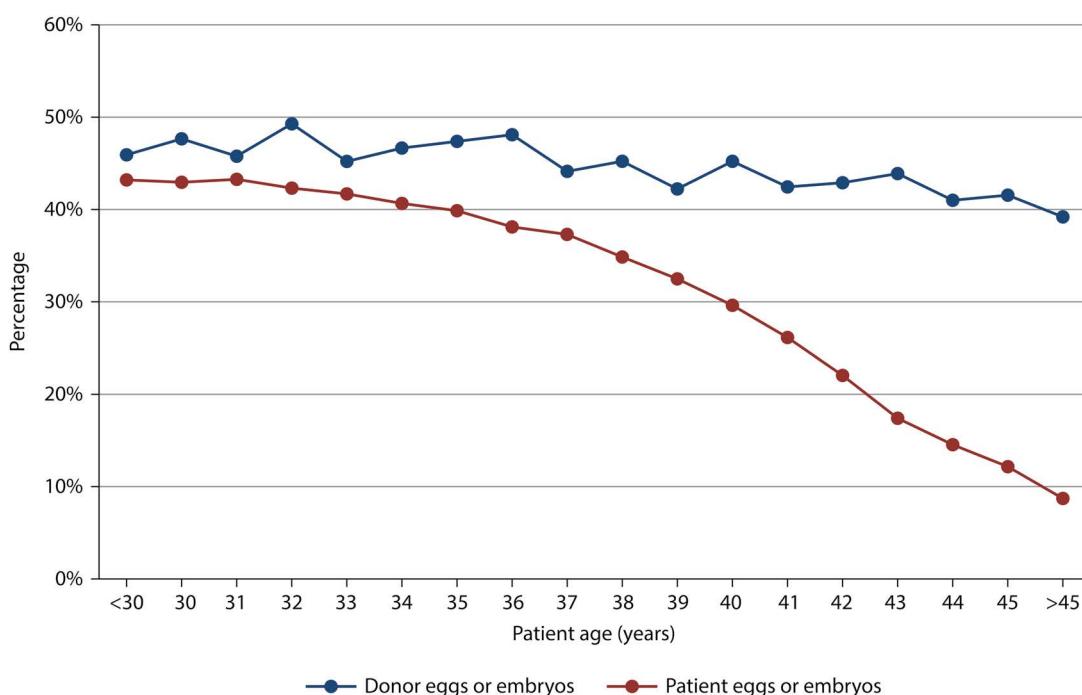


FIGURE 71.1 Percentage of embryo transfers that resulted in live-birth delivery, by patient age and egg or embryo source, United States, 2019. (2019 National Summary Report on ART success rates, CDC: <https://www.cdc.gov/art/artdata/index.html#reports?>)

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

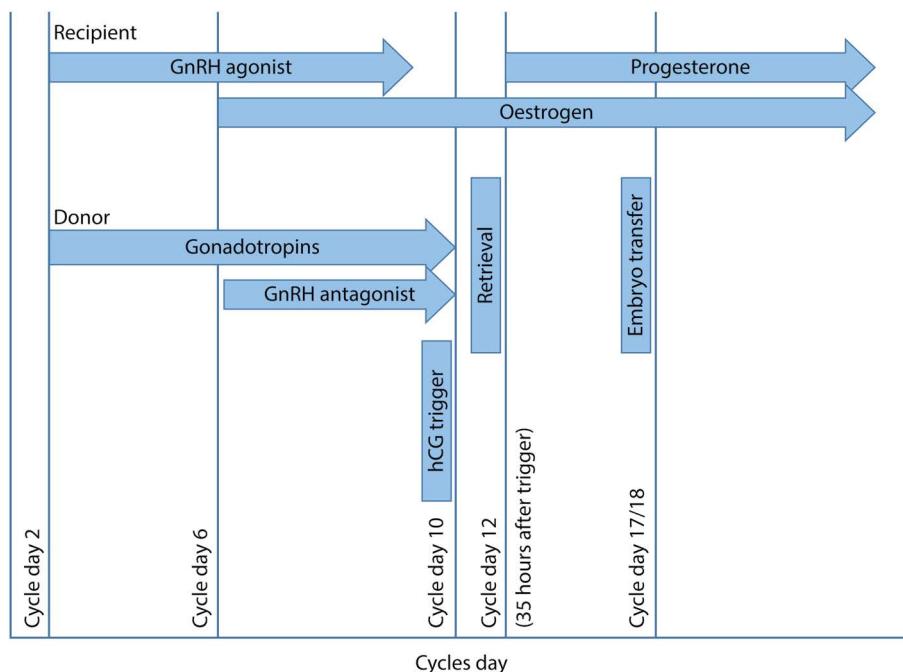


FIGURE 71.2 Schematic representation of cycle synchronization using a GnRH agonist in both donor and recipient. GnRH agonists are used to downregulate the pituitary of recipients with evidence of ovarian activity prior to beginning oral oestradiol. Oral oestradiol is prescribed to the recipient four 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.

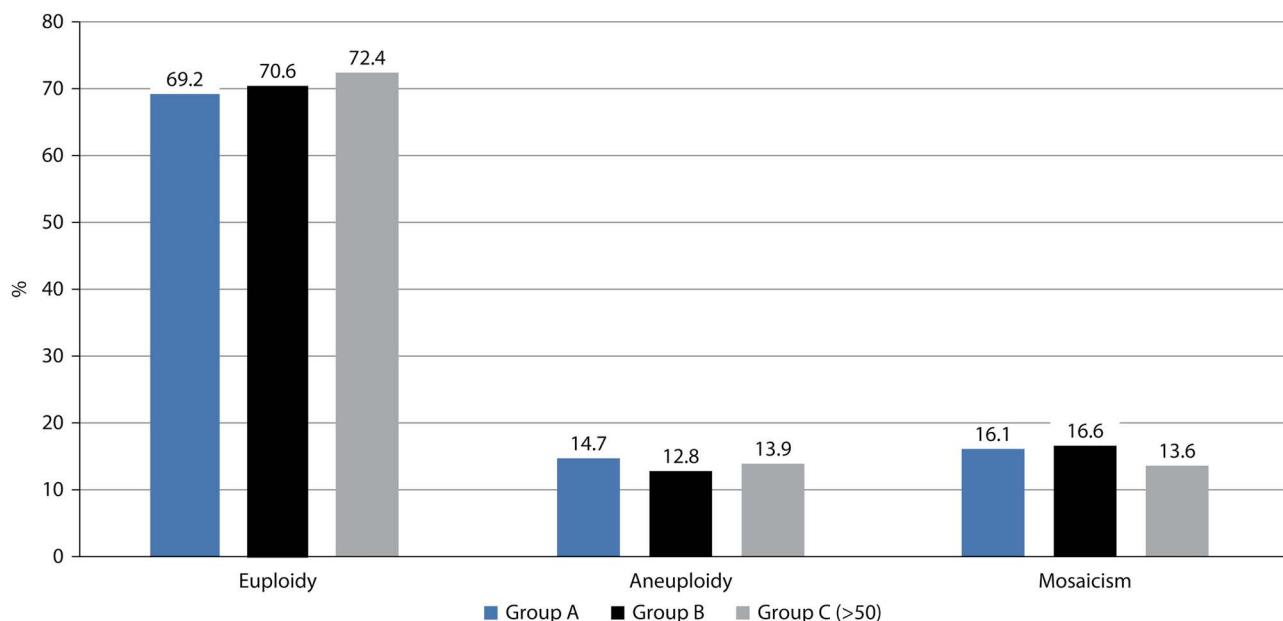


FIGURE 71.3 Ploidy rates for assisted reproduction technology cycles using PGT-A tested embryos from donor eggs, by male partner's age, 2020: paternal age group A (blue) ≤ 39 years ($n = 592$); B (black) 40–49 years ($n = 496$); C ≥ 50 years ($n = 97$). (From [69], with permission.)

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.

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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.

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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.

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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.

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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].

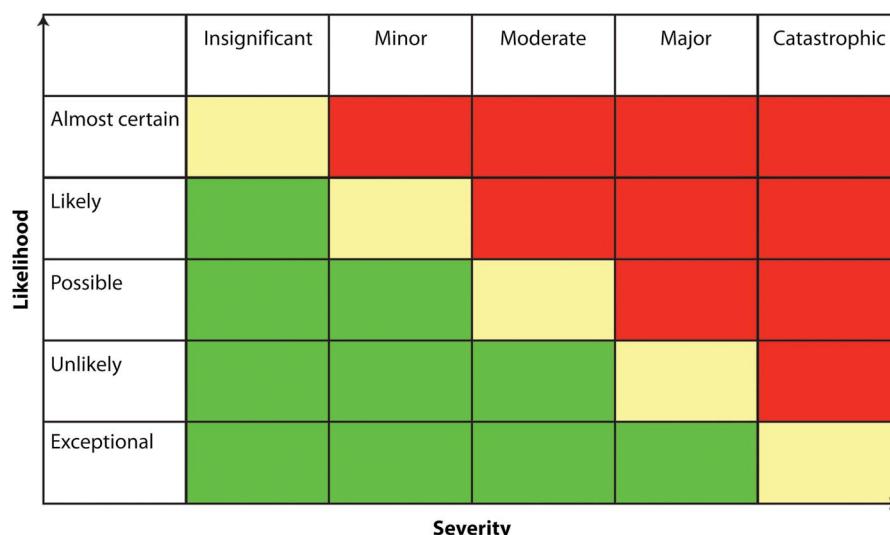


FIGURE 75.2 Know your risk. The consequences of any given risk result from a dynamic process that can be plotted on a likelihood versus severity diagram. In the case of post-oocyte retrieval haemorrhage, a slight increase in intrauterine bleeding (incident) may progress towards a dramatic, uncontrolled, 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.

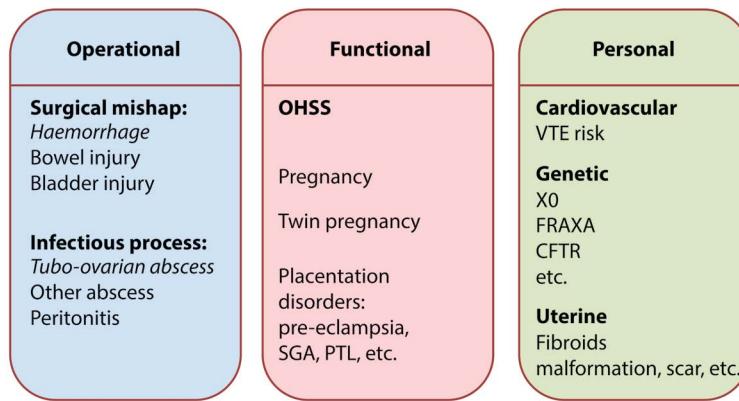


FIGURE 75.3 Three types of risk in assisted reproduction technology. Abbreviations: OHSS, ovarian hyperstimulation syndrome; PTL, preterm labour; SGA, small for gestational age; VTE, venous-thromboembolism.

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

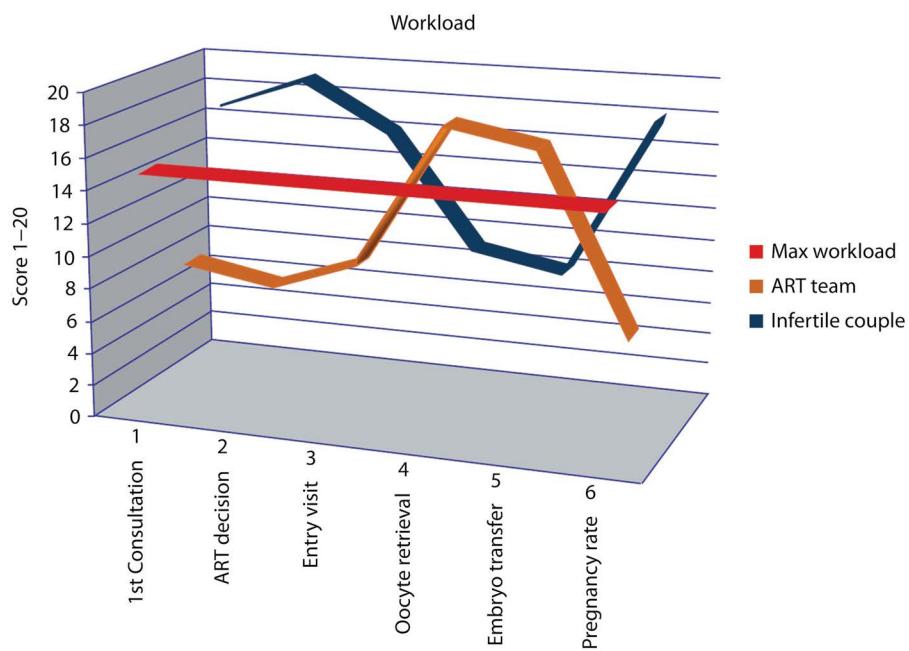


FIGURE 75.4 Workload pattern throughout the ART process. Workload increases at certain times in the ART process, and these increases are different for the various members of the medical teams. Cycle synchronization—using OC or other means—can help with avoiding reaching an excessive workload for team 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.

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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
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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

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