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Assisted reproductive techniques: risks, contraindications, prognostic factors, therapeutic strategies

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Therapeutic approaches involving assisted reproductive techniques (ART) are aimed at achieving a high ongoing pregnancy rate with a low rate of multiple pregnancies, ovarian hyperstimulation syndrome (oHSS) and other undesirable side effects. Indications for conventional *in vitro* fertilization (cIVF) therapy are fallopian tube factor, failure of previous *in vivo* regimens, risk of multiple pregnancies in an *in vivo* gonadotrophin cycle, and also the rarely occurring luteinized unruptured follicle syndrome. Indications for an intracytoplasmic sperm injection–*in vitro* fertilization (ICSI–IVF) trial are OAT syndrome III°, use of fresh or thawed/ cryopreserved samples from testicular sperm extraction (TESE), OAT syndrome I–II° after 3–6 unsuccessful cycles with intrauterine insemination, fertilization failure in a previous cIVF cycle, linear progressive sperm motility <15% and normal sperm morphology <3% as determined by electron microscopy. Relative or absolute contraindications are patients >44 years, menopausal transition or menopause, anomalies of the Müllerian duct system, Asherman syndrome, multiple leiomyoma uteri (surrogate motherhood is not allowed in Germany), oestrogen resistance (OR) of the endometrium (<5.0 mm), meno-metrorrhagia, unclear ovarian cysts, status following cancer of the internal genital tract or the breast, acute or subacute infections of the internal genitals, diseases that are not compatible with carrying a baby to term, severe systemic-progressive, genetic, neurotic or psychotic diseases, use of teratogenic drugs, abuse of tobacco, alcohol or any kind of drugs, and social evils. The prospective view may be helpful for a qualified management of treatment. Accordingly, the putative response (OR) may be subdivided into three groups: low, good, and high OR. The definition of a good OR is applicable if the following clinical and laboratory features are found: number of follicles >5<12 without the presence of small follicles, E2 concentrations >1000<3000 pg ml^{-1} (prior hCG administration), number of oocytes around 10, transfer of two good-quality 8-cell embryos on day 3 (day 0 : day of egg retrieval); this group comprises patients <37 years who have an exclusive fallopian tube factor (cIVF), and/or OAT syndrome III° (ICSI–IVF), a good OR in the pre-cycle as well as after pregnancy. The high OR group, which is associated with a high risk of oHSS after hormone stimulation and/or induction of pregnancy, consists of patients with ovaries having bilaterally >10 follicles and with E2 concentrations >3000 pg ml^{-1} (prior hCG); this group comprises patients <35 years, with body mass index (BMI) <23 kg/m^2, hypo-, normogonadotrophic amenorrhea, polyfollicular ovary II–III° (PCOS) and patients who had >3 dominant follicles in preceding *in vivo* cycles. The low OR group consists of patients with <5 dominant follicles, E2 concentrations <1000 pg ml^{-1} (prior hCG), <5 oocytes per retrieval and <2 embryos with good cleavage rate and morphology; this group comprises patients >36 years, FSH >10 mU ml^{-1} (day 3–6 of the cycle), status after ovarian cystectomy (e.g. endometrioma), status after

unilateral oophorectomy, or status after <5 oocytes retrieved in the preceding cycle. Taking together, major predictors of the ART outcome are age, cycle pattern, BMI, sonographic morphology of the ovaries, sperm quality, and response in preceding *in vivo* or *in vitro* cycles. In order to prevent oHSS in presumably high OR cycles, a safe approach in such ART cycles is recommended: follicle stimulating hormone dosage <200 IU per day, discontinuation of stimulation if there is an excess of both ovarian follicles and E2 concentrations; hCG administration at a lower dominant follicle size (>14<17 mm), a lower hCG oocyte maturation dosage of 4000 IU, cryopreservation of 2-PN cells (and of embryos) after oocyte retrieval without embryo transfer in the current cycle, no hCG administration in the luteal phase, intravenous infusion of macromolecules around and after follicular retrieval. In optimally managed ART trials, the cryopreservation programme is an important supporting method, particularly in the special German situation where embryo freezing is prohibited by law. Use of thawed/cryopreserved 2-PN cells is associated with an increased pregnancy rate/follicle retrieval, fewer transfers of fresh embryos <3 in the current cycle, and therefore, prevention of multiple pregnancies and oHSS. Helpful modalities are cryopreservation of spermatocytes in TESE/MESA and a 'security deposit' of cryopreserved spermatocytes for a planned ICSI–IVF trial in patients with severe OAT syndrome III°. The CGE & RMF data of 2002, which have to be evaluated under the principles of a prospective management, are as follows: ongoing pregnancy rate/embryo transfer: 29%; number of cycles with cryopreservation of 2-PN cells: 66%; twin pregnancies: 6%, no triplets; abortion rate/pregnancy: 6%; pregnancy rate/embryo transfer using embryos from thawed/cryopreserved 2-PN cells: 18%. These data show that by using a prospective moderate approach in ART cycles, a cumulative pregnancy rate/oocyte retrieval of 40% with a low rate of twin pregnancies (and without triplets) may be achieved – even under the strict German legislation.

Limits of infertility surgery – gynaecological view

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Introduction. In 1994, R.D. Kempers in his function as editor-in-chief, wrote in a leader that 'surgical reconstruction of the reproductive tract either laparoscopically or by laparotomy will soon become a thing of the past' (Kempers, 1994). It should be remembered that in 1994 *in vitro* fertilization (IVF) was already a highly developed technique and intracytoplasmic sperm injection (ICSI) had become routine procedure for the treatment of subfertility in men. In a matter of some years only few indications for primary microsurgical correction actually remained. On the other hand, certain surgical techniques became generally recognized as adjunct measures prior to the commencement of assisted reproduction. This was especially true in women with hydrosalinges, endometriosis, ovarian cysts and myomas. In such cases it seems that preceding infertility surgery can indeed lead to better results in assisted reproduction.

Fertility surgery as a primary procedure. Microsurgery as a 'first line' therapy is always indicated if there is a favourable ratio of intrauterine pregnancies (IUP) to extrauterine pregnancies (EUP). Such a positive constellation is to be found with regard to microsurgical ovariolysis and salpingolysis on patent oviducts, with an IUP/EUP ratio of 33 to 2% (16.5). An equally positive relationship exists concerning the status after tubal sterilization, with an IUP/EUP ratio of 89 to 8% (11.1). These results are more successful than in conventional IVF treatment, which shows an IUP/EUP ratio of 35 to 5% (7.0) per trial. Other forms of infertility surgery such as fimbrioplasty or anastomosis in proximal tubal occlusion are less effective than IVF treatment and have a high EUP rate of 10–20%. A particularly unfavourable result is produced by microsurgical salpingostomy of peripherally occluded oviducts, which has an IUP/EUP ratio of only 1.6. Unfortunately, distal tubal occlusion is the most frequent form of post-inflammatory tubal damage so that this pathological condition represents a common indication for IVF. However, women with hydrosalpinges have bad chances of success in the IVF programme. This fact increases the importance of infertility surgery as an adjunct procedure.

Infertility surgery as an adjunct procedure. *Hydrosalpinges*: For several years, there have been publications about the negative impact of hydrosalpinges on the IVF programme. According to a meta-analysis conducted in 1998, the success rate of implantation and pregnancy concerning women with hydrosalpinges was approximately 50% lower than average (Zeyneloglu *et al.*, 1998). Another analysis published in 1999 came to the same conclusion. Thus it would seem that pregnancy, implantation and birth rates can decrease up to 50% in the presence of hydrosalpinges, while at the same time the number of early loss of pregnancies

also increases (1.7-fold) (Camus *et al.*, (1999). In a controlled and randomized prospective study, we were able to confirm the benefit derived from the microsurgical correction of hydrosalpinges through salpingostomy, prior to commencing assisted reproduction techniques (ART). In 20 pre-operated women with the correction of their hydrosalpinges, 11 (55%) became pregnant after the first IVF cycle; in women with nonoperated hydrosalpinges, pregnancy occurred in just one out of 20 cases (5%). It was evident that a significantly improved ART result could be achieved by undertaking the surgical correction of bilateral and ultrasonographically detectable hydrosalpinges (Table 1). Up to now there have only been hypotheses about the pathogenetic effect of hydrosalpinges in IVF. Discussion in this respect focuses on the one hand, on purely mechanical factors such as outflow of the embryo from the cavum uteri and on the other hand on reduced receptivity of the endometrium. A more recent idea suggests that the reduction in the implantation rate may be caused by a hydrosalpinx-related increase in pressure in the fundus area, with an adverse transport of liquid and particles in the direction of the cervix uteri.

Endometriosis: Severe endometriosis is one of the most common indications for IVF. In the meantime, however, there is increasing evidence that endometriosis may itself negatively influence the outcome of ART. A meta-analysis carried out in 2002 confirmed that the odds ratio for an IVF pregnancy was reduced to 0.81 in women with endometriosis as compared to women without endometriosis (Barnhart *et al.*, 2002). In addition to a reduction in the pregnancy rate, there was also a significant reduction in the implantation rate. Our own studies concerning endometriosis and assisted reproduction concentrated on the question whether infertile women with endometriosis profit from a combination of infertility surgery and medication-based endometriosis therapy. In a controlled and randomized prospective study, we demonstrated the beneficial effect of long-term gonadotrophin releasing hormone (GnRH) analogue (GnRH-A) therapy for 6 months prior to ART in cases of advanced endometriosis (Rickes *et al.*, 2002). The advantages of long-term GnRH-A therapy are manifold and multifactorial. The GnRH-A-related hypoestrogenism would imply an atrophy of the endometriosis. The downregulation of the gonadotrophins leads to a WHO-I constellation with an increase in the pregnancy rate. Leutinizing hormone reduction is of great benefit to all women in whom such concentrations are pathologically elevated. Finally, we would like to mention that an amenorrhoic uterus is especially suitable for the implantation of embryos. Besides all these general advantages, GnRH-A therapy also leads to local improvements in the endometriosis condition. For example, deletal factors such as IL-1 and TNF are suppressed, while as endometrial receptivity returns to normal, natural killer cells can function again and apoptosis is reactivated.

Ovarial cysts and myomas: The presence of ovarian cysts during the IVF cycle has a negative effect on the overall result. In these cases ovarian stimulation is always accompanied by low oestradiol concentrations, higher incidence of abortion, and a smaller number of oocytes; consequently, implantation and pregnancy rates are reduced. All myomas, no matter where they are located, impair uterine perfusion and implantation, thus causing the abortion rate to rise. As myomas lead to a reduction in pregnancy and birth rates after IVF, their initial enucleation by means of laparoscopy or laparotomy is indicated.

Conclusion. In view of the present-day possibilities offered by assisted reproduction techniques, infertility surgery is limited to just a small number of applications such as adhesiolysis and refertilization. Its real value in relation to IVF therapy is summed up by the motto 'prepare the ground properly before beginning the fertilization process'.

Table 1. ART pregnancy rates in hydrosalpinges subgroups

Condition	Corrected HSX	Persistent HSX	*P*-value
Bilateral HSX, *n*	16	15	
PR/pat., *n* (%)	15 (93.7)	6 (40)	0.002
US-visible HSX, *n*	13	14	
PR/pat., *n* (%)	12 (92.3)	5 (35.7)	0.003
Bilateral + US-HSX, *n*	9	11	
PR/pat., *n* (%)	8 (88.9)	4 (36.4)	0.025

ART = assisted reproduction techniques; HSX = hydrosalpinges; PR = pregnancy rate; US = ultrasonography; US-HSX = ultrasonographically visible hydrosalpinges; Pat. = patient.

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Pre-implantation genetic diagnosis – possibilities and pitfalls

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Pre-implantation genetic diagnosis (PGD) is not new: already Gardner & Edwards (1968) published a successful PGD for sex selection by detecting the sex chromatin in rabbit pre-implantation embryos. The first PGD in humans was published by Handyside *et al.* (1990): the authors performed PCR of a specific genomic sequence of the Y-chromosome for sex selection of pre-implantation embryos in a couple with an X-linked inherited disease. After 2 years, the same authors reported the first successful PGD for a specific mutation (ΔF508) in a couple with both parents being carriers for the same mutation (Handyside *et al.*, 1992).

Pre-implantation genetic diagnosis had initially been developed to help couples at risk to inherit a specific disease onto their offspring, to conceive a healthy child. This was achieved by detecting those pre-implantation embryos that did not carry the mutation and by selecting those embryos for transfer in an *in vitro* fertilization (IVF) attempt. Methodologically, this became possible by examining either polar bodies obtained at the zygote stage, or one or two single blastomeres obtained at the 8- to 12-cell stage, either by PCR mostly for specific mutations, or by fluorescence *in situ* hybridization (FISH) mostly for numeric chromosomal abnormalities. Since then, PGD has been performed worldwide in a vast variety of indications. In 1997, the European Society for Human Reproduction and Embryology (ESHRE) founded the ESHRE PGD Consortium, which collects data from 25 centres performing PGD all over the world. The data are published on a yearly basis and an overview of the reasons for PGD is presented in Table 1. According to the data published, PGD had been performed on 14 040 pre-implantation embryos in these 25 centres (ESHRE PGD Consortium Steering Committee, 2002).

Basic concerns against PGD are a possible destruction of pre-implantation embryos by the blastomere biopsy and the possibility of misdiagnoses caused by allelic drop-out or mosaicism of the different blastomeres within one embryo. So far, the blastomere biopsy procedure has been shown to be safe: in an animal model, Hardy *et al.* (1990) have shown that removal of one, two or three blastomeres from 8-cell rabbit embryos adversely affected the further development of the pre-implantation embryo. Furthermore, the ESHRE data show that 13 689 of 14 040 biopsied embryos (98%) survived the biopsy procedure (ESHRE PGD Consortium Steering Committee, 2002).

Table 1. Reasons for preimplantation genetic diagnosis (PGD)

Reason for PGD	Number of cases
Aneuploidy (advanced maternal age, recurrent miscarriages):	799
Sex determination (in cases of X-linked diseases like DMD, haemophilia, fra-X):	294
Cystic fibrosis (mostly ΔF508 mutation):	109
Translocations	368
Autosomal dominant diseases (Chorea Huntington, Marfan-S., osteogenesis imperfecta)	254
Autosomal-recessive diseases (ß-thalassemia, sickle cell anemia, Tay-Sachs, Rh incomp.)	290
Social sexing	78

Several cases of misdiagnoses by PGD have been reported and the rate is somewhere around 3.5% (ESHRE PGDS Consortium Steering Committee, 2002). These misdiagnoses are mostly caused by allelic drop-out or preferential amplification of a single allele during PCR of a single cell, but increased sensitivity and specificity of the methodology, mostly by introduction of fluorescent PCR, have reduced this rate. Also, simultaneous detection of the allele of interest for PGD and other alleles allow to detect allelic drop-out by internal control for plausibility. Another reason for possible misdiagnoses is mosaicism within the single blastomeres of an embryo. Munne *et al.* (1994) showed that even in morphologically normal human pre-implantation embryos that were considered 'good quality embryos' by criteria of light microscopy, the rate of mosaicism was around 18% when chromosomes X, Y and 18 were screened by FISH. This mosaicism rate increased to 28% when 'bad quality embryos' were examined for the same chromosomes. These findings have resulted in the recommendation to biopsy and examine two blastomeres from an 8-cell stage pre-implantation embryo and to only use the results for diagnosis when both blastomeres show the same pattern.

Other reasons for PGD besides the detection of affected embryos, in cases of parents with a known risk to inherit a specific disease, are the screening

for aneuploidy in cases of multiple implantation failures in patients undergoing IVF or in couples who suffer from recurrent miscarriages. It is a well established fact that the pregnancy rate in IVF dramatically decreases with advanced maternal age. From oocyte donation programmes it became apparent that this decrease is not caused by a less receptive endometrium, but by decreased oocyte and embryo quality (Navot *et al.*, 1994). This is because of meiotic failure of the oocytes from older women. A case-controlled study by Munne *et al.* (1999) showed that, after PGD for aneuploid embryos from older women and by selective transfer of euploid embryos, the pregnancy rate was higher and the rate of miscarriages was significantly lower after PGD. Other reasons for research on pre-implantation embryos include the need for better knowledge of the physiology of pre-implantation embryo development and embryonic implantation, to possibly increase the pregnancy rates in human IVF therapy.

The situation in Germany is dominated by the embryo protection act (Embryonenschutzgesetz, EschG) of 1991, which does not allow destruction of pre-implantation embryos and of totipotent embryonic cells. The question whether or not a single blastomere from an 8-cell pre-implantation embryo is totipotent or not, cannot be answered with the current state of knowledge. Interestingly, research on this question is against the law in Germany. Using a mouse model, Piotrowska *et al.* (2001) showed that differentiation into later embryoblast and later trophoblast does exist as early as in 2-cell-embryos, giving rise to the assumption that in 8-cell embryos totipotency is no longer existent. The EschG, however, does not specifically prohibit polar body biopsy for PGD and several centres in Germany have reported first experiences with this method. The pitfall of polar body biopsy for PGD is that only maternal genetic material can be detected, thus limiting the method mostly for aneuploidy screening. The inability to detect paternal alleles inside the polar bodies does not allow to use this methodology in cases of paternally inherited diseases. Another limitation of the EschG is the maximum number of three embryos per IVF cycle. Data from the ESHRE PGD Consortium Steering Committee (2002) clearly states that the number of embryos for PGD should be around eight in order to have a sufficient number of nonaffected embryos for transfer. Even if PGD might basically be introduced in Germany after some parliamentary debates, the limitation to three embryos will prove to be absolutely impractical and will have to be changed as well.

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Assisted reproduction: genetic aspects – risk of malformations – pre-treatment counselling

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It is more than 10 years now that intracytoplasmic sperm injection (ICSI) was added to the therapeutic armamentarium of human reproductive medicine. Nevertheless, researchers, clinicians and patients alike are still perplexed by the question, whether or not ICSI is genetically safe. Moreover, fertility clinics apply widely divergent strategies in pre-treatment genetic counselling and testing for their patients. Some have no form of genetic review at all, some use and even mandate an extensive pre-treatment workup. The German public health system recently issued guidelines advocating routine pre-treatment genetic counselling for all patients intending to make use of ICSI.

Until now, there is no generally accepted approach to pre-ICSI genetic counselling and testing. In the author's experience a three-step scheme has proved useful. First, through documenting the general medical history, reviewing the reproductive data (e.g. results of semen analysis), and drawing the family pedigree, possible genetic risk factors are sought. In the second step, it ensues a discussion of these data and their risk-relevance with the patients. Closely interwoven with this, genetic laboratory tests are planned and initiated as necessary (e.g. karyotyping, DNA tests). Also, the most pertinent data about genetic effects and risks of the ICSI technique *per se* are reviewed with the couple. In the majority of cases, these first two steps can be performed in a single, 45–90 min session. As the third and final step, some time after the counselling session when the laboratory has returned the test results, a written summary and expertise is sent both to the couple and to the clinicians involved. If karyotyping or other tests have yielded abnormal results a second appointment with the geneticist ensues.

It is not the aim of genetic counselling and testing to exclude patients from treatment against their will. If substantial genetic risks are involved, the couple may still desire to take their chances and embark upon treatment. In fact, most couples with a genetic risk factor do so, some with the intent to use pre-natal diagnosis to cover the problem, if applicable. This notwithstanding, every physician has the right to reject active participation in a treatment where a severely handicapped child is a more or less likely outcome. In such a situation, it may be difficult to strike a balance between the highly-valued reproductive autonomy of the infertile couple and the desire of the physician not to embark on a genetically hazardous treatment.

More often than genetic risks are found they can be excluded. As a matter of fact, the most common and very important result of genetic counselling and testing prior to ICSI is reduction of patient anxiety. Many couples view themselves as not being destined by 'mother nature' to become parents. These and similar ideas revolving around poor semenograms (especially teratozoospermia), recurrent pregnancy losses and other reasons for involuntary childlessness, are largely mythical and poorly fact-based. It is a worthwhile activity for the counsellor to invest a bit of time to discuss these issues in a calm atmosphere, often with the effect to relieve patients from an enormous amount of – objectively irrational – fear and anxiety.

The use of cost-intensive genetic laboratory tests should be tailored to each couple's individual needs. In the author's opinion not every patient heading for ICSI has automatically to be karyotyped, although this is certainly the test that should be applied most generously. Indiscriminant testing of all male ICSI candidates for Y-microdeletions and mutations in the cystic fibrosis gene CFTR is clearly unnecessary. Studying the published literature allows for a rational selection of patients for these and other tests, and for avoiding unnecessary costs. A detailed description of indications for the various genetic laboratory tests is beyond the scope of this paper.

What should patients be told about the genetic safety of ICSI? (1) It is clear now the the rate of malformations is moderately increased. In the worst case, as suggested by an Australian study (Hansen *et al.*, 2002), the risk for a physical abnormality may be doubled against the population baseline. Other publications show a less pronounced increase in the malformation rate. (2) The risk for a chromosomal abnormality is also increased, probably to a level of around 1–2%. (3) Data about psychomotor development of ICSI children are scant. One report claimed poorer early performance of ICSI children, but this was not independently confirmed and may have been because of problems in study design. A recent study from the UK showed no difference in early psychomotor development between ICSI- and naturally-conceived children (Sutcliffe *et al.*, 2001). (4) Children born after ICSI probably are at increased risk for fertility problems later in their lives – simply because of the fact that all their parents are affected.

Table 1. Summary of genetic risks possibly associated with ICSI

Type of abnormality	Frequency	Quality of evidence
Major malformation	Frequency increased. Current maximum estimate: doubling against baseline frequency in the local population.	Solid
Chromosomal aberration	Increased to a rate of 1–2% (at time of amniocentesis)	Preliminary
Retardation of early psychomotor development	???	Weak/controversial
Infertility in adult age	???	No empirical evidence – purely hypothetical
Imprinting defect	???	Very preliminary

Recently a new and rather disquieting facet of the possible genetic risk spectrum of ICSI has emerged. Small case series suggest that ISCI could sometimes interfere with a process termed genomic or parental imprinting (Orstavik *et al.*, 2003; Cox *et al.*, 2002; DeBaun *et al.*, 2003). This epigenetic modification is of great importance for the function of certain genes which display a parent-of-origin specific pattern of expression. If ICSI does have the potential to adversely affect imprinting, disorders like Angelman syndrome and Beckwith-Wiedemann syndrome could be more common than in the general population.

In view of the genetic risks inherent in ICSI, all women pregnant through this technique should have access to invasive pre-natal diagnosis (e.g. amniocentesis), high quality ultrasound, and other advanced techniques of pre-natal medicine. Table 1 summarizes the most pertinent data about genetic risks possibly associated with the use of ICSI.

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From the oocyte to the blastocyst

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The primary aim of *in vitro* fertilization (IVF) treatment is not only to achieve a pregnancy, but also to offer the best modalities to fulfil the wish of the future parents for a healthy child. Therefore the transfer of only one embryo with the highest implantation potential should be mandatory, as every multiple pregnancy can be considered as a pregnancy at risk. Increasing knowledge about how to identify this single embryo is available, based on consecutive classifications at each step of early embryo development, starting with the oocyte and ending at the blastocyst stage.

Unfortunately, the best means to identify the one embryo with the highest implantation potential is not always possible, especially when legal restrictions exist which only allow for a selection at the pronuclear stage, as in Switzerland and Germany. Some strategies which are in agreement with the strict legal guidance of the German and Swiss embryo protection law will be outlined in this short manuscript.

Since 1998, detailed morphological criteria are available for the classification of oocytes with two pronuclei (Scott & Smith, 1998). These are mainly based on the number and local distribution of the so-called nucleolar precursor bodies (NPB) within the pronuclei of the fertilized oocyte. The classification led to the definition of specific pronuclear morphology patterns, some of which exhibited better embryonic development and higher blastocyst formation rates.

Consequently, a German multicentric study revealed higher pregnancy and implantation rates, following selection of good prognosis pronuclear morphology patterns compared with others (Montag & van der Ven, on behalf of the German Pronuclear Morphology Study Group, 2001). This led to the conclusion that, under the strict guidelines of the German embryo protection law, patients will benefit from pronuclear scoring as this offers the only possibility to legally choose at the very beginning of development those cells with a higher potential for future embryo development and implantation.

The multicentric study also revealed that in women at advanced age (>35 years), oocytes with a pronuclear pattern with a good prognosis were less frequently available than in younger women. This observation raised the question of the scientific background of pronuclear morphology. Based on cytological investigations, the structures within the pronuclei were termed NPB, which represent the nucleolar organizer regions located on chromosomes 13, 14, 15, 21 and 22. However, it cannot be excluded that these structures are the result of an associaction of centromeric regions of all chromosomes.

Although the scientific discussion has not come to an end, it is clear that at least some chromosomes are involved in the formation of the NPB. Therefore, it is interesting to know if the morphological appearance of the NPB is somehow linked to chromosomal aneuploidies and if certain pronuclear morphology patterns are found more often in aneuploid cells compared with others.

Chromosomal aneuploidies frequently occur in woman above 35 years of age. In Germany, the only way to reveal the presence of aneuploidy in oocytes is polar body diagnosis. We recently obtained ethical approval to perform polar body biopsy and to investigate the nature of pronuclear morphology patterns in aneuploid oocytes (Montag *et al.*, 2002). Our preliminary data shows that pronuclear morphology patterns with a good prognosis, regarding future embryo development, are to the same extent euploid or aneuploid. In other words, a selection based on pronuclear morphology does not allow for an exclusion of chromosomal aneuploidies for patients at risk to have chromsomal aneuploidies.

Therefore, we speculated if a combination of pronuclear scoring and investigations on aneuploidy would enhance the outcome of IVF treatment, especially in women over 35 years. Such a strategy is under investigation in our IVF clinics. Preliminary results were obtained from 100 patients and these clearly show a benefit for those patients in whom euploid pronuclear stage oocytes derived from good prognosis were available for later transfer.

Based on these results, we would propose to investigate the benefit of this strategy for a larger cohort of patients in a controlled multicentric study. The resulting data will then allow to set up general guidelines for the best treatment of this group of patients under the German embryo protection law.

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Is there an increased genetic risk of old fathers towards their offspring?

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While an increased incidence of chromosomal anomalies in children from mothers of advanced age has undoubtedly been proven, the influence of advanced paternal age has by far not been so well investigated. In principle, the following genetic alterations have to be considered: numerical chromosomal anomalies, structural chromosomal anomalies and autosomal dominant mutations.

Aneuploidy resulting from numerical chromosomal aberrations is mainly because of a nondisjunction during meiotic division of the gametes. Aneuploidy is the most frequently detected chromosomal abnormality in human liveborns and spontaneous abortions. Aneuploid spermatozoa may be observed in men at puberty and are found in 5% of healthy men, whereas their number is significantly increased in infertile men. Fortunately, the results of earlier studies, demonstrating an increased occurrence of trisomy 21 (Down syndrome) in children of fathers over 5 years, were not confirmed by later investigations. In addition, there was no relationship between the incidence of clinically relevant chromosomal aberrations for trisomy 13, trisomy 18 and Turner syndrome (45,X0) and paternal age.

Paternally derived errors account for only 10% of most autosomal trisomies (rarely for trisomy 16), whereas in Klinefelter patients, the incidence of paternal errors in 47,XXY individuals is approximately 50% with no relation to the age. In summary, most studies provide no evidence of a paternal age effect for numerical chromosomal aberrations. Only very recently a centromeric deletion of chromosome 1 showed a positive association with age.

Structural chromosomal anomalies do, however, seem to be more frequent in spermatozoa of older men. Martin & Rademaker (1987) observed the highest incidence of structural chromosomal anomalies in men over 44 years, with approximately 13% of spermatozoa showing chromosomal damage. Using fluorescence *in situ* hybridization (FISH) the authors confirmed a positive age effect for structural abnormalities only. However, so far there has been no evidence from studies in live newborns or prenatally diagnosed fetuses that older fathers have an increased frequency of offspring with *de novo* (noninherited) structural chromosomal anomalies including inherited or unbalanced Robertsonian translocations.

Most important are genetic mutations resulting from errors in DNA replication, which have to be considered as a possible cause of genetic risk factors, as they can be passed onto the next generation as a single gene defect. An increased number of spontaneous mutations in older men had been suspected long ago, first time by the German physician and obstetrician Weinberg in 1912, but only recently evidence has shown that an increased risk to develop achondroplasia and Apert syndrome was

because of paternal age. There is a dramatic increase of the spontaneous mutation rate with a factor of 10 when fathers at an age of 20 years are compared with those of 40 years. However, data are not sufficient to generalize a negative paternal age effect concerning point mutations to all autosomal dominant disorders (e.g. myositis ossificans, Marfan syndrome, neurofibromatosis, polycystic kidney disease, poliposis coli and progeria). In addition, a paternal age effect acting at the level of the maternal grandfather has been suggested for X-linked recessive disorders like hemophilia A and Lesch–Nyhan syndrome.

An increased chance of error in DNA transcription obviously occurs in men because of remarkable differences between spermatogenesis and oogenesis. DNA replication and mitotic cell divisions of primordial germ cells in females get terminated in utero after approximately 22 cell divisions, whereas in males this process continues age-dependently throughout the reproductive life. For example, in a 20-year-old male the number of premeiotic cell divisions is approximately 150, compared with 840 in a 50-year-old men. Each mitosis involves the risk of spontaneous point mutations. An increased number of *de novo* mutations result from a paternally derived age-dependent increase of DNA base exchanges.

By means of molecular biology, it is possible to determine whether a mutation occurred in the mother or in the father. It has been shown that sporadic cases of several autosomal dominant diseases result primarily from a DNA base exchange in the paternal germ line (e.g. achondroplasia, Apert syndrome or multiple endocrine neoplasia type 2) and that affected persons had older fathers than the normal population. In contrast, other mutations, such as intragenic microdeletions or microduplications are less dependent on paternal ageing.

In sporadic neurofibromatosis type 1, only one-third of the mutations are DNA base substitutions, with a strong paternally derived excess and age-dependent increase of cases, whereas intragenic deletions have been found more frequently in the maternal germ line.

The slight paternal age increase observed in neurofibromatosis and Duchenne muscular dystrophy is not comparable with the large increase found for achondroplasia and Apert's syndrome. A possible explanation is that Duchennes dystrophy and neurofibromatosis are caused by very large genes with many introns. Deletions or duplications of a few hundred or thousand of DNA bases are in this case less likely to be lethal, than if this occurred in a smaller gene.

In summary, there are enough clinical data for several autosomal dominant disorders which support that point mutations occur much more often in males and that there is a large paternal age effect on their offspring. In contrast, deletions and duplication do not show an age effect and the rate, if it is indeed different in the sexes, is greater in females.

Apart from the above mentioned 'copy error' model as an explanation for the exponential increase of DNA base substitutions in the paternal germ line, other possibly relevant hypotheses include age-related impairment of DNA repair mechanisms, age-related suppression of apoptosis following oxidative stress and age-related decrease in the activity of antioxidative enzymes in seminal plasma and spermatozoa.

Concerning diseases with a complex etiology, it is difficult to evaluate a paternal age effect. The reproducibility of results in several studies has to be considered, especially in sporadic cases. The data are conflicting concerning Alzheimer's disease or cardiovascular malformations like heart septal defects. In some studies, a paternally derived age effect was found concerning sporadic schizophrenia. Without the detection of the relevant susceptible gene or several genes this finding is not conclusive. For several malignancies such as acute lymphoblastic leukaemia, Wilms tumour or prostate cancer, insufficient data exists concerning a paternally derived age effect.

In summary, from the mentioned findings an increased incidence of certain genetic diseases in the offspring caused by spontaneous locus mutations within paternal chromosomes seems to be evident of old fathers. In addition, it has been shown that the proportion of abortions after the 19th week of gestation increases continuously with increasing paternal age, independently of maternal age (Hook, 1986). Of these, possibly a significant proportion may be associated with fresh dominant mutations.

In conclusion, even if children of older fathers have an increased incidence of certain genetic diseases, the individual risk can be considered very low. Therefore, genetic counselling regarding the risk of older men to have children with an autosomal dominant or X-linked genetic disorder is questionable. This is in accordance with guidelines of the German Federal Medical Board (1998) stating that advanced paternal age in contrast to advanced age of pregnant women is not an explicit indication for intensified pre-natal diagnosis.

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Current aspects of donor insemination

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Heterologous or donor insemination is a treatment option for unvoluntarily childless couples. The legal term 'heterologous' characterizes the fact of non-identical social and genetic paternity. The term 'donor' is derived from the medico-psychological point of view of 'offering, donating and helping'. Thus, artificial donor insemination (ADI) is recommended for the practice of reproductive medicine.

In Germany, this specific treatment is performed in about 35 medical centres and since the 1970s about 50 000 children have been born after such a procedure. Indications are severe male infertility and high genetic risks for a child. The ADI is an alternative for infertile couples when all other methods of 'homologous' systems with husband spermatozoa failed to achieve pregnancy, including intracytoplasmic sperm injection (ICSI) with ejaculated spermatozoa or after testicular epididymal sperm extraction (TESE). Relative indications are refusal or inadequately high risks of a therapeutic testicular biopsy. Furthermore, ADI may be considered for patients after cancer chemotherapy without prior sperm cryopreservation, vasectomized men who cannot be refertilized and for those in whom expensive ICSI or adoption are unrealizable.

Selection of a sperm donor requires high semen quality, e.g. normozoospermia as defined by the WHO, and cryotolerance. The health check should include a risk assessment for hereditary diseases (medical history, clinical examination and facultative genetic testing), exclusion of infections (HIV, HBV, HCV, CMV, syphilis, gonorrhoea, chlamydia and toxoplasmosis) and quarantine banking of semen for 6 months before use. It is recommended to select the donor with regard to body features, blood groups and rhesus factor. In order to minimize the risk of incest in half-siblings born after ADI, the number of pregnancies is limited to 10 per donor. The ADI treatment requires gynaecological pre-screening and monitoring of the cycle. The cumulative pregnancy rate after intracervical insemination is 50–65% and comparable with that of a normal healthy population (Fig. 1). Abortion rates, risk of ectopic gravidity, birth weight, premature births, sex ratio, frequency of congenital defects, postnatal development and socioemotional development of the child are within normal range (Krause, 1999; Golombok *et al.*, 2002). Several follow-up studies regarding the psychosocial impact of ADI, on the parents, showed no significant alteration and decrease in quality of life. The low divorce rate of 2% and the frequent request for repeated ADI are indicative of successful treatment. Whether or not the child should be informed about his/her origin is controversial: 8.6–30% (median 21%) want to inform their child, 54–80.2% (median 75%) reject to do so. Reasons for leaving the child uninformed are reluctance to admit infertility, insecurity about when and how to give such information and the psychological impact on the child concerning the lack of knowledge about the genetic father.

The development of the legal and medico-legal framework regarding ADI in Germany since 1970 shows that this treatment is legally and ethically accepted and can be performed in the countries of the European Community. However, the German law for protection of embryos ('Embryonenschutzgesetz') of 1990 does not contain any regulations. According to the 1994 guidelines for assisted reproduction, heterologous insemination is allowed, provided that the following criteria are considered: specific indication, prohibition to pool spermatozoa from different donors, right to claim paternity and right of the child to know his/her genetic father. The reform of parapraph 1600

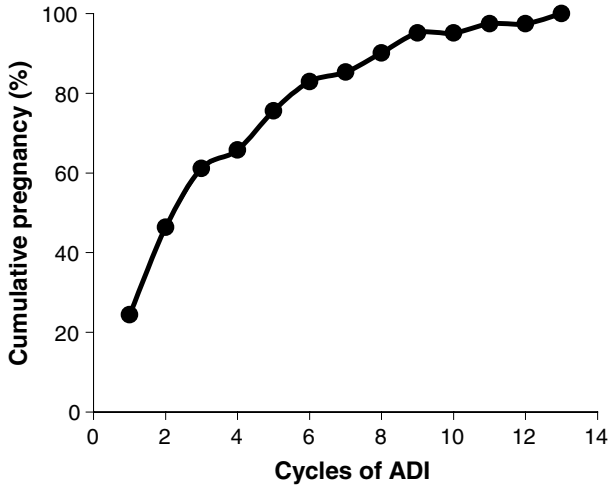

Figure 1. Cumulative pregnancy rate after donor insemination is comparable to that in a normal population.

BGB (German Civil Law) in April 2002 is a step further in favour of ADI. The standards for this treatment and the guidelines for cryobanking of semen are published by the Society for Donor Insemination.

In Germany, the donor has to face legal risks, in that his fathership can be identified. Theoretically, the right of a father–child relationship has to be considered, e.g. the right to visit the genetic father or questions of heritage (Coester-Waltjen 2002).

Physicians are not obliged to perform ADI, they do this treatment voluntarily. For security, they should strictly adhere to the following modalities: information of both the patient and the donor, additional legal advice, declaration of consent by the couple before a notary, documentation, no promise of anonymity towards the donor, avoidance of pooled semen samples, adequate time of document storage. However, further legal regulation concerning ADI modalities in Germany are required.

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Limits of microsurgical refertilization under urological aspects

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Vas deferens obstructions can be differentiated into epididymal obstruction, obstruction of the deferent duct, and central obstruction. Etiologically, all three groups may have congenital, inflammatory or iatrogenic causes.

Microsurgical vasovasostomy (VV) is the most frequently performed procedure for refertilization. Its chances of success in terms of pregnancy results are partly dependent on the interval between vasectomy and VV (Belker *et al.*, 1991). While a pregnancy rate of 25% is still achieved after obstruction intervals of more than 25 years, the patency rate remains virtually unaffected (Fig. 1) (Fuchs & Burt, 2002). Therefore, long obstruction intervals seem to be no obstacle to VV. If the female partner is older than 40 years, VV is superior to intracytoplasmic sperm injection (ICSI) (pregnancy rate 15% versus 4%), as shown by a comparative study (Fuchs & Burt, 2002). Another unusual situation is refertilization after childhood herniorrhaphy. Despite the fact that more than 80% of anastomosis sites are located at the internal hernial ring or retroperitoneum, acceptable patency and pregnancy can be achieved, 44 and 36%, respectively (Matsuda, 2000). Refertilization operations on larger defects in the vas are seldom performed, so that only single case reports are available for evaluation. The cross-over technique used is always individually adapted. Failure of VV repeatedly raises the question of whether another refertilization operation or microsurgical epididymal sperm aspiration (MESA) should be performed. According to a few studies on larger number of patients (Belker *et al.*, 1991; Paick, 2000), repeated VV (patency rate 75–91.9%, pregnancy rate 43–57.1%) is clearly superior to the MESA/ICSI procedure.

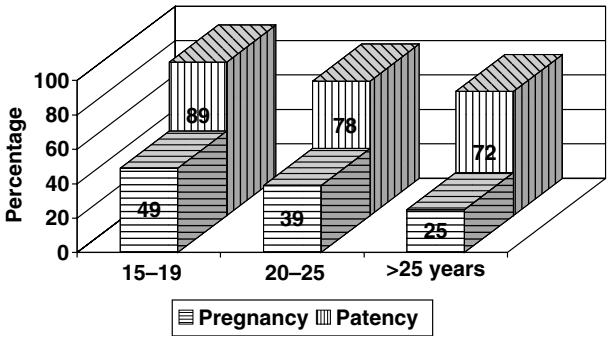

Figure 1. Patency and pregnancy rates depending on obstruction intervals (according to Fuchs & Burt, 2002).

The limits of operability in cases of tubulovasostomy are determined by the degree of tubular obstruction. Even with maximal microscopic magnification and finest thread material, an unobstructed tubule cannot be safely anastomosed to the deferent duct. The success in terms of pregnancy outcome depends on the site of epididymal anastomosis and decreases towards the epididymal head (Weidner *et al.*, 1995).

Transurethral resection of the ejaculatory ducts (TURED) is seldom performed. Our own studies (Schroeder-Printzen *et al.*, 2000) and a MEDLINE research over a period of 20 years have shown that cystic lesions have a significantly higher success rate than noncystic obstructions. As the prostate and upper efferent ducts run within the vicinity of the rectum, resection requires particular care.

In summary, it can be stated that VV is superior to all other procedures in cases of long obstruction interval, advanced female partner age, and reversal. The surgical possibilities of performing tubulovasostomy or TURED are limited by the degree of obstruction and the anatomical situation.

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Therapeutic strategies in severe male factor infertility

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During the last decade, modern techniques of assisted reproduction have profoundly changed the management of male factor infertility. Notably, intracytoplasmic sperm injection (ICSI) has proved to be an effective procedure and is considered to be the method of choice for cases with severely impaired semen quality. In contrast, conventional treatment of male infertility is regarded to be of little value and has been largely neglected. This may be because of the lack of controlled clinical studies meeting the criteria of evidence-based medicine and providing conclusive data on the efficacy of a given therapeutic regimen (Haidl, 2002). Assessment of conventional therapeutic strategies is hampered by the multifactorial aetiology of male infertility and the related heterogeneity of patient subpopulations. In at least 25% of subfertile men, more than one causal factor can be identified (Rowe *et al.*, 2000). On the other hand, in up to 40% of cases the aetiology of disturbed fertility remains unclear. Moreover, the occurrence of pregnancy in the partner as preferred end-point is influenced by female factors, whereas semen parameters show great physiological variability. Despite these drawbacks, barren couples should not be referred to ICSI programmes without prior andrological examination of the male partner. Detailed diagnostic work-up allows identification of causal factors of impaired semen quality, including those accessible to appropriate treatment or specific recommendations. This approach is highly recommended even in men with severe disturbances, such as extreme oligoasthenoteratozoospermia or azoospermia.

Environmental factors may cause male infertility or contribute to its severity, in conjunction with preexisting genetic disorders or diseases of the genital organs. In this context, however, patients seeking medical intervention often disregard potentially harmful lifestyle factors, such as genital heat stress, tobacco smoke, alcohol, or pharmaceuticals, which should be eliminated. Concerning the latter issue, abuse of anabolic steroids is well known to have detrimental effects on male reproductive functions. Alarming figures indicate that abuse is not restricted to athletes and bodybuilders but represents a major health risk in the general population including adolescents. High doses of multiple compounds are often administered in repeated courses over years, resulting in complete testicular failure, i.e. azoospermia (Gazvani *et al.*, 1997). In some cases semen quality does not return to normal values, following the cessation of abuse, and hormone replacement therapy may be required to re-initiate spermatogenesis. Similarly to the therapy of idiopathic hypogonadotrophic hypogonadism and Kallmann syndrome (Haidl, 2002), administration of gonadotrophins (hCG, hMG) proved to be effective.

Infection and inflammation of the male reproductive tract are widely accepted as important etiological factors of male infertility (Rowe *et al.*, 2000). Chronic asymptomatic inflammation can be a major cause of impaired semen quality, particularly when the epididymis and/or the testis are involved (Schuppe *et al.*, 2001; Haidl, 2002). However, diagnostic criteria are not sufficient and the impact of genital tract infection, and inflammation on sperm function and fertilization remains a matter of debate. On the other hand, several reports have indicated that antibacterial and/or nonsteroidal antiphlogistic therapy may improve

semen quality, even in patients with azoospermia or extreme oligoasthenoteratozoospermia (Martin-Du Pan *et al.*, 1997; Montag *et al.*, 1999; Haidl, 2002). According to our own observations, anti-inflammatory treatment is also a promising approach if no viable spermatozoa are present in the ejaculate (necrozoospermia) because of asymptomatic inflammation and dysfunction of the epididymis. Thus, retrieval of testicular sperm, as recommended by Tournaye *et al.* (1996), can be avoided in selected patients.

Genital tract inflammation may also accompany other pathologies, such as testicular damage after cryptorchidism or hemodynamically relevant varicoceles. In these cases, anti-inflammatory treatment represents a first-line therapeutic option (Rowe *et al.*, 2000). In contrast, management of a varicocele is a highly controversial issue (Haidl, 2002). Especially in patients with a multifactorial aetiology of their infertility, the impact of a varicocele is often neglected. Some reports, however, indicate that varicocele treatment may have beneficial effects even in men with elevated follicle stimulating hormone levels, reduced testicular volume, and azoospermia (Matthews *et al.*, 1998; Kruse *et al.*, 1999). These observations illustrate that appropriate therapeutic strategies allow significant improvement of semen quality in individual patients. Subsequently, less invasive methods of assisted reproduction may become applicable (Haidl, 2002).

Management of severe male factor infertility should include a thorough search for testicular tumours. Compared with samples from the general male population, the prevalence of carcinoma *in situ* (CIS) as precursor of testicular cancer is increased in infertile men, especially those with a history of undescended testes or testicular atrophy and extremely low sperm counts (Rørth *et al.*, 2000). Before treatment of CIS or testicular neoplasia, patients should be offered cryopreservation of semen samples whenever possible. In cases of azoospermia or extreme cryptozoospermia, cryopreservation of testicular tissue for sperm extraction is recommended. This approach proved to be successful in a patient with the rare condition of bilateral CIS. ICSI could be performed with testicular sperm obtained from residual foci of spermatogenetic activity in one of the testes, which resulted in an ongoing pregnancy and birth of a healthy child. Spermatozoa for ICSI were also retrieved from tumour-free biopsies of a patient with metachronous bilateral testicular cancer, in whom frozen semen samples were not available (Köhn *et al.*, 2001). However, data reflecting potential risks in sons from fathers who underwent sperm retrieval from neoplastic testes for ICSI, are not available.

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Cryopreservation of ovarian tissue to preserve female fertility – state of the art

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Introduction. New and often aggressive treatment schemes allow the successful healing of many young patients with cancer, most of whom suffer from lymphatic diseases. But the price the young women have to pay is high: many of them lose ovarian function and fertility. Oncologists gradually develop an understanding that hormone replacement therapy is not enough for the patients.

While chemotherapy using antimetabolites induces ovarian failure only in a few percentage of the patients, the risk after doxyrubicine, mitoxantrone or platinum is between 30 and 60%. Cyclophosphamide or busulphan induce ovarian failure in 70% of the patients, and after high-dose chemotherapy and total body irradiation, about 92% of the patients are menopausal.

The patient's age is of major importance: while young women with mammary cancer seldom suffer from ovarian failure after chemotherapy, the rate rises to nearly 100% in patients over the age of 40.

Methods to preserve fertility. Since the 1990s, good success has been reported in ovarian protection using gonadotrophin releasing hormone agonists. It has been proposed that these substances act by blocking the hypophyseal secretion of luteinizing hormone and follicle-stimulating hormone, thereby inhibiting the proliferation of granulosa cells and follicular development. Unfortunately, there are no major randomized, prospective studies that would show the effectiveness of this treatment.

An alternative is the cryopreservation of female gametes or ovarian tissue. While freezing of gametes is routinely used worldwide within *in vitro* fertilization and intracytoplasmic sperm injection programmes, this is not an option for the majority of the cancer patients. In most of the cases, there is no time for stimulation, no partner available, or the patient does not feel healthy enough to undergo the procedure. Moreover, unfertilized oocytes are difficult to handle during freezing. Although the method has been used since 1984, only 60 children have been born worldwide since then.

To avoid these problems, some teams are working on cryopreservation of ovarian tissue, which has up to several thousand follicles per mm^2. To date, however, it has not been possible to keep thawed tissue alive for an extended period of time, neither *in vivo* nor *in vitro*.

State of the art. Though with low success rates, it was possible to produce living offspring in mice after transplantation of thawed ovaries to recipients. In sheep, normal ovarian function was achieved after transplantation of frozen/thawed ovaries.

Our own data shows that most of the ovaries, but only 40% of intact follicles, survive freezing (Siebzehnrübl *et al.*, 2000). Xenotransplantation of parts of thawed human ovaries to severe combined immunodeficient mice was successful, but only a small part of the tissue was functional afterwards. As early as 1996, it was shown that lymphoma can also be transmitted (Shaw *et al.*, 1996). Therefore, much work has to be done before a clinical study is possible.

After thawing, *in vitro* culture of cryopreserved human ovarian tissue failed to produce follicles beyond the state of primordial follicles.

Summary. Cryopreservation of ovarian tissue before chemotherapy and/or radiation for cancer is an interesting, but clinically not relevant option to preserve fertility of female cancer patients. However, considering the effect of highly aggressive treatments, this is the only chance for these women. Many teams are working on solutions for the present problems, worldwide. Although it is not possible to guarantee the success of cryopreservation, at present this treatment offers the patients a possibility to retain fertility, which is of great importance, and if only for psychological reasons.

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Limitations for ICSI, MESA, TESE? – experiences from the IVF centre in Giessen

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The pregnancy rate in modern reproductive medicine depends on several factors. One of them is the quality of gametes used in an IVF cycle. Which quality parameters are required for successful insemination? Since the method of intracytoplasmic sperm injection (ICSI) was established in the early 1990s, the traditional andrological sperm parameters like sperm count, motility and morphology have lost their importance. Now, at least one single spermatozoon and one oocyte are required to reach fertilization and to achieve pregnancy. Nevertheless, there are some criteria that might influence the success of the therapy.

An important criterion is the vitality of the spermatozoa. Staining methods to identify vital and dead sperm cells are not suitable during an ICSI procedure. Normally, sperm motility is the most important sign for vitality. In some cases, no motile spermatozoa can be detected. Use of the hypo-osmotic swelling test (Ved *et al.*, 1997) has

been suggested for ejaculates with 100% asthenozoospermia. The method is easy: because of the hypo-osmotic solution, vital spermatozoa with an intact membrane show swollen flagella. Unfortunately, the assessment is difficult in ejaculates with extremely poor sperm number or in biopsies of testicular tissue. Such specimens show spermatozoa embedded in dendritus and other particles, which could impair the analysis. Therefore, motility is the most important criterion for ICSI.

Why is it important to have vital sperm in case of ICSI? Dead spermatozoa exhibit disturbed membrane integrity, the membrane could have 'leaks'. This might cause damage to the DNA in the sperm head, e.g. because of reactive oxygen species. Furthermore, cytoplasmic factors may be lost, which are important for the fertilization reaction. This could explain the apparent contradiction that no immotile sperm should be used for ICSI, but motile spermatozoa are immobilized directly before injection into the oocyte. It is desirable to release this factor, but only directly after injection into the ooplasm. The significance of this fact is shown in Table 1. In ICSI cycles with only immotile sperm available, we observed a strongly decreased fertilization rate (52.5 versus 78.8%) and implantation rate of the resulting embryos (15.1 versus 31.6% pregnancies per embryo transfer).

Other criteria for sperm quality are genomic factors, such as aneuploidies or DNA fragmentation. A study by the Institute for Reproductive Medicine and the Centre of Andrology in Giessen (Henkel *et al.*, 2002), demonstrated a strong and significant correlation between the percentage of sperm with DNA fragmentation (detected by TUNEL assay) and the resulting pregnancy rate. Cycles with a low number of sperm DNA fragmention were compared to those with a high number of such disturbances. Despite the significant difference in the pregnancy rate, the fertilization was not influenced. However, DNA fragmentation cannot be identified during an ICSI procedure. Only in cases of sperm with cytoplasmic retention, a significant correlation to aneuplodies was demonstrated (Kovanci *et al.*, 2001). It is not clear at the moment whether disturbed chromatin condensation has an influence on ICSI results.

Table 1. Comparison of ICSI cycles with motile and immotile spermatozoa. Despite the low number of cycles with 100% immotile sperm, fertilization rate, embryo score and pregnancy rates are much lower than in the other group. Female age and number of transferred embryos were not significantly different in both groups

	ICSI (motile sperm)	ICSI (100% immotile sperm)
Cycles	2717	79
Fertilization	78.8%	52.4%
Embryo score	14.0	9.3
Pregnancies/embryo transfer	31.6	15.1%

Table 2. Influence of motility in testicular and epididymal sperm. In most cases of epididymal aspiration, at least a small number of motile sperm could be found. The results of these cycles are comparable with those obtained from motile testicular sperm (data not shown). Therefore, cycles with motile epididymal and testicular sperm were summed. Although immotile testicular spermatozoa are mostly vital, the ICSI results were significantly lower. Female age and number of transferred embryos were not significantly different in both groups

	MESA/TESE motile sperm	TESE immotile sperm
Cycles	130	131
Fertilization	75.6%	52.7%
Pregnancies/embryo transfer	32.0%	10.8%
Birth rate/embryo transfer	22.3%	5.2%

Spermatozoa from testicular tissue are mostly immotile, but 90% of them are vital (Schulze *et al.*, 1999). Therefore, testicular sperm should be able to fertilize oocytes. Table 2 shows the experience of the MESA/TESE group in Giessen, with testicular and epididymal sperm. In a comparison between cycles with motile epididymal or testicular sperm and cycles with only immotile testicular sperm used for ICSI, decreased fertilization (52.7 versus 75.6%) and pregnancy rates (10.8 versus 32.0%) in case of lacking motility, were demonstrated. Whereas the results with motile sperm are similar to those with ejaculated sperm, the success rate is much lower in testicular tissue without motile sperm. This could be an indication for an unknown factor of sperm maturity. The results of ICSI with round cell spermatids emphasize this finding. Although some pregnancies after injection of round cell spermatids have been published, the results remains poor (Levran *et al.*, 2000).

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Perspectives in the diagnosis of testicular biopsies using molecular biological techniques

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The evaluation of a testicular biopsy should, (i) confirm or exclude carcinoma *in situ* (CIS) and (ii) determine the effectiveness of spermatogenesis, in order to clarify whether an infertile man can become father by application of testicular sperm extraction (TESE) and following intracytoplasmic sperm injection (ICSI). While the immunohistochemical detection of placental alkaline phosphatase (PlAP) is routinely used for identification of CIS, no reliable factor exists so far that could predict the outcome of TESE/ICSI. However, such a factor is urgently required, as selection of a 'looser spermatozoon' has both financial and emotional consequences for the couple.

To date, several factors including follicle-stimulating hormone (FSH) have been demonstrated to have no effect on the outcome of TESE/ICSI (for review see Steger, 2001). However, it is well known that infertile men exhibit reduced chromatin condensation, caused by incomplete histone-to-protamine exchange in haploid spermatids (for review see Steger, 2001). Interestingly, the protamine-1 to protamine-2 ratio seems to have more influence on male fertility than the absolute quantity of protamines. Spermatozoa from infertile men have been found to exhibit a decreased protamine-1 and protamine-2 protein content and, in addition, an aberrant protamine-1 to protamine-2 protein ratio (Balhorn *et al.*, 1988). Data obtained on the protein level were confirmed on the mRNA level, applying *in-situ* hybridization followed by quantitative analysis of the percentage of protamine-positive round spermatids in testicular biopsies (Steger *et al.*, 2001). While spermatozoa from infertile men that revealed a decreased but equal distribution of protamine-1 and protamine-2 in round spermatids, were able to fertilize an oocyte, spermatozoa failed to fertilize when round spermatids in testicular biopsies exhibited an aberrant protamine-1 to protamine-2 mRNA ratio. This implies that the protein content of spermatozoa can be predicted by analyzing the mRNA equipment of their progenitor cell, namely round spermatids in testicular biopsies. This phenomenon is caused by chromatin condensation resulting in the stop of gene expression in haploid spermatids, which prevents the addition of new genetic information.

Therefore, use of molecular biological techniques in the evaluation of testicular biopsies will, in future, contribute to obtain much more information about the patient. Both the patient and his descendants will benefit from this improved diagnosis. To reserve a maximum of technical possibilities, testicular biopsies should be divided into two halves. While one part is fixed in Bouin and embedded in paraffin serving for histological evaluation, *in-situ* hybridization, and immunohistochemistry, the other part is cryofixed in liquid nitrogen serving for reverse transcription and both normal and quantitative PCR.

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Reproductive medicine after menopause

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Family planning has revealed profound changes in the last decades. Delayed parenting is increasing with an average age of primiparous women in Germany of 29 years.

This behaviour might be related to late marriage, higher grade of female education, increasing female employment and an increasing number of divorced couples.

More than 10% of German women in *in vitro* fertilization (IVF) programmes are older than 40 years. Nevertheless, female age is the most decisive fertility factor.

The German IVF register shows a pregnancy rate of 31.37% in women under 30 and a decline to less than 10% in women over the age of 43. Therefore, assisted reproductive techniques might not be indicated to overcome reduced fertility by female age.

Since the physiological ovarian reserve is almost exhausted in periclimacteric women, the only option would be oocyte donation.

Oocyte donation allows extremely high pregnancy rates of up to 40%, even in women after natural menopause. Sauer *et al.* (1993) reported eight clinical pregnancies after 21 embryo transfers in women with an average age of 50 years with only one abortion.

However, older women have an increased risk of gestational diabetes, pre-eclampsia, placenta praevia, premature rupture of membranes and a higher rate of caesarian section.

For example, among 103 clinical pregnancies, Sauer *et al.* (1996) found nine premature deliveries, eight patients with hypertension and two patients with pre-eclampsia (Table 1 for details).

In order to allow an ethical evaluation of oocyte donation, natural menopause must be discriminated from diseases that might lead to hypergonadotrophic ovarian insufficiency. The latter group comprises POF syndrome, gonadal dysgenesia, chromosomal aberrations and iatrogenic manipulations, i.e. irradiation, chemotherapy, radical surgery. In particular, for these patients, oocyte donation must be demanded to allow at least biological motherhood.

Oocyte donation after natural menopause has severe ethical concerns. There is no good definition, as far as the age of 'natural menopause' is concerned.

The German Society of Gynaecological Endocrinology and Reproductive Medicine favours a limit of 45 years.

Furthermore, social difficulties for a child with very old parents and the actual live expectancy of the presumptive parents must be taken into account. Finally, the real motivation of an unusually old couple for oocyte donation must be deeply evaluated.

If assisted reproductive techniques will be extended far beyond medical and physiological borders, patients will definitely demand the use of these techniques for their own social and nonmedical purpose. Cryopreservation of oocytes in younger age and their use after menopause will be a realistic model in the near future for some people, but this is not what reproductive medicine was made for.

In summary, oocyte donation might be a good choice for many patients with premature ovarian insufficiency. After natural menopause, it is not a medical indication at all, it does not cure anything, but is a definite misuse of modern achievements of assisted reproduction. Therefore, the new campaign of the American Society for Reproductive Medicine 'Advancing age decreases your ability to have children' is of utmost importance.

Table 1. Pregnancy and pregnancy outcome after oocyte donation (Sauer *et al.*, 1996)

No. of patients	162
Age (years)	47.3 (45–59)
No. of transferred embryos	4.5 ± 1.1
Clinical pregnancies	103
Abortions (17 biochem. pregnancies)	29
Live births	74
Premature deliveries	9
Multiple pregnancies	29 (39.2%)
Pregnancy-induced hypertension	8
Pre-eclampsia	2
Gestational diabetes	6
Maternal death	0
HELLP syndrome	6
Foetal growth retardation	2
Down syndrome	1
Small bowel atresia	1
Heart failure	1

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Gynaecological malignancies and infertility

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Introduction. When a malignoma is diagnosed, the immediate question is how to proceed further. This question involves uncertainty regarding the mode and radicality of therapy (surgery, chemotherapy, radiotherapy). It is also the imperative demand for likelihood of survival and mode of survival.

With increasing survival rates, especially in early-stage gynaecological tumours, the issue of quality of life becomes more and more important and also involves family planning. In young women without a

definite partner infertility has to be taken as seriously as in married couples who want to have a baby.

Any therapy should be aimed at achieving a very high standard of anti-tumour effect, which is least harmful in terms of quality of life, including maintenance of fertility.

Cervical carcinoma. As in cases of *in situ carcinoma*, a cone biopsy is fully sufficient. It is important that traumatization of the biopsy area is kept as low as possible, i.e. only little coagulation, thermocoagulation rather than electrocoagulation, or even preferably laser cone biopsy. Sturmdorf sutures should be avoided.

In *carcinomas of the cervix*, radical trachelectomy offers the chance to maintain the corpus uteri, thereby allowing pregnancy (Dargent *et al.*, 2000).

Inclusion criteria: Young women with early-stage invasive cervical carcinoma, no positive lymph nodes, extremely careful and precise counselling of the patient so that she is fully aware of the operation and its risk.

Method: Lymphonodectomy (para-aortal and pelvic with frozen section, to exclude metastases).

Vaginal approach: Circular incision of the vagina for creation of a vaginal cuff, resection of bladder pillars, rectum pillars and descending branches of uterine artery. Resection of uterus 5 mm below isthmus, followed by isthmic cerclage and anastomosis of vagina and isthmus.

Results: Recurrence 4%, pregnancy in 50%, infertility in 30%, radiotherapy 16%, abortion rate 25%.

Breast cancer. Roughly 20% of breast cancers occur in women at reproductive age. It is estimated that this percentage is increasing as the age of primiparae is increasing as well. With advancing age of primiparae, the number of infertile women will increase as the risk of breast cancer also rises with advancing age. Primary concern of breast cancer in pregnancy is stimulation of 'sleeping micrometastases' and increase in carcinogenesis by elevated levels of endogenous pregnancy hormones and growth factors. However, poor data are available, and these are mainly retrospective. Basically, good survival chances have been observed in pregnant women following breast cancer. Of utmost importance is the time interval between the end of therapy and the onset of pregnancy. If pregnancy occurs 6 months after therapy, the 5-year survival rate is 53.8% compared with 78%, if pregnancy begins 2 years after the end of therapy. All studies on pregnancy and breast cancer have shown that a longer interval between the end of therapy and the onset of pregnancy resulted in a better survival rate.

Concerning amenorrhea after adjuvant chemotherapy, gonadotrophin releasing hormone analogues have been discussed as protective agents for the ovaries. Amenorrhea has frequently been observed after tamoxifen therapy.

Ovarian carcinoma. *Borderline carcinoma*: Once a borderline carcinoma has been established, a representative biopsy should be taken from the contralateral ovary. In case of uncertainty, a second-opinion consultation is advisable. In principle, organ-preserving surgery should be performed. Controls are recommended at short intervals until family planning has been successful, then radical surgery is advised. In case of established *ovarian carcinoma* and desire to have children, normal radical surgery including para-aortal lymphadenectomy, frozen section omentectomy, appendectomy, unilateral ovarectomy and contralateral ovarian biopsy should be performed. If possible, the contralateral ovary and the uterus should remain. If pregnancy occurs 1 year following chemotherapy for ovarian cancer, the risk of genetic malformations is not increased. However, a higher rate of small-for-gestational-age children has been observed.

Conclusion. Once gynaecological malignancies are followed by pregnancy, the future of the mother remains uncertain; however, in most cases it would be positive. Consequently, the duty of the gynaecologist is to explain all details of therapy, including survival rates. A decision in favour of a pregnancy and therefore parenthood, is entirely the patient's or the couple's matter.

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Epididymal sperm retrieval: indications, risks, and outcome

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In azoospermic patients without disturbed spermatogenesis, the following indications for epididymal sperm retrieval techniques are generally accepted: congenital aplasia of the vas deferens (CBAVD), correctable obstruction of the seminal pathways, ejaculatory disorders where conservative therapy has not been successful, and in particular, failed vasovasostomy (Weidner *et al.*, 2002).

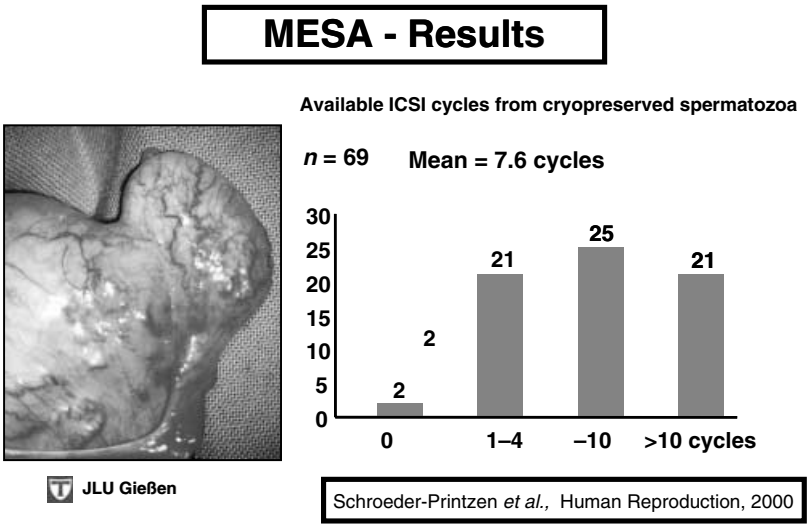

Figure 1. Available ICSI cycles from cryopreserved spermatozoa.

Microsurgical epididymal sperm aspiration (MESA) is the therapy of choice and MESA combined with intracytoplasmic sperm injection (ICSI) has increased the number of pregnancies. The fertilization rate is not affected by the nature of obstruction (congenital or acquired) and can be as high as 90% per cycle. At present, pregnancy rates of between 14 and 66% per cycle are achieved when MESA is combined with ICSI.

The synchronized combination of MESA and ICSI requires a large number of logistic facilities and involves hormonal stimulation of the female partner pre-operatively, even though it is not certain at this point whether spermatozoa will be retrievable at all. When epididymal aspiration with subsequent cryopreservation is dissociated from ICSI, hormonal stimulation of the female is started, after it has been clarified that enough motile spermatozoa are available for ICSI. There are no data in the literature that show a significant difference in clinical pregnancy rates when using fresh or cryopreserved epididymal spermatozoa. Damage to the spermatozoa caused by cryopreservation seems to be tolerable and the dis-synchronized MESA/ICSI procedure is the procedure of choice of our Giessen MESA/TESE group.

Successful sperm retrieval is based on an optimal microsurgical technique. It seems clear that the largest number of motile spermatozoa are found in the caput of the epididymis. There is only limited information on the number of vials taken for ICSI and the quality of the cryopreserved spermatozoa, in the literature mentioned above. In our hands, the available ICSI cycles from cryopreserved spermatozoa range between 1 and 10 cycles (Fig. 1). So far only Oates *et al.* (1996) have reported on a similar amount of cryopreserved material.

In conclusion, our data demonstrate MESA as a successful procedure to retrieve spermatozoa in 94% of all patients with obstructive azoospermia. We consider MESA as a standardized procedure for epididymal sperm retrieval.

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HIV-infected people who wish to have children – chances and limits of assisted reproductive techniques

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During the past few years, in Western industrial countries, the reality of HIV infection has undergone a considerable change with regard to life expectancy and feasible life plans. Because of the fact that the majority of the HIV-positive population is of reproductive age, one of these life plans can be to have children. The need for medical

support to realize this wish, however, is often confronted by ethical, medical or forensic reservations.

Care for and support of HIV-positive people who wish for a child is an interdisciplinary task. Should the situation arise, also nonmedical psycho-social offers have to be integrated. Basic elements in counselling of the couples are disease progression, early and comprehensive infectiology diagnosis as well as fertility screening of both partners. All steps of diagnosis and treatment have to be documented completely, as well as information about the remaining risk of infection for the healthy partner or, possibly, the child. In cases of lack of compliance in one partner or advanced stage of HIV-disease (CDC B3 or C), active reproductive support should not be carried out (Weigel *et al.*, 2001).

But those who seek for our advice are particularly couples who want to handle their HIV infection responsibly and who do not wish to evade the issue of unprotected sex with the healthy partner. In principle, HIV discordant couples, who desire children, present with the following two scenarios:

- If the male partner is HIV-positive, one only has to deal with preventing infection of the healthy female partner.
- If the female partner is HIV-positive, one is not just concerned with preventing infection of the healthy male partner, but also with preventing infection of the child. In addition, possible interactions between HIV infection and pregnancy have to be considered.

None of the studies in HIV-positive women published to date have shown that pregnancy and birth adversely affect the course of a nonadvanced HIV infection. However, infection does seem to increase the probability of complications arising during pregnancy. Without medical intervention, the risk of viral transfer from mother to child is about 15–20%, mainly determined by the course of delivery. Current standard recommendations for reducing the risk of materno-fetal transmission include anti-retroviral therapy during pregnancy, a neonatal anti-retroviral prophylaxis, abstention from breast feeding, and elective caesarian section before onset of labour (Buchholz *et al.*, 2002). By performing all these recommendations, the foetal risk of infection can be reduced to about 1%.

If the couple decides to persue their family planning after a thorough consultation, an early fertility and infection check of both partners should be performed. If any barriers to fertility can be excluded, HIV-positive women should be informed about the possibility of self-insemination, which protects the healthy partner from HIV-infection. Medical measures taken to reconstruct fertility or to optimize conception are acceptable as long as polyovulations are prevented. Techniques of assisted reproduction in HIV-positive women, however, should be done with strong reservations, the well-being of the future child being given highest priority by the current guidelines – at least in Germany. The remaining risk of materno-fetal transmission, which under optimized conditions is low but still quantifiable, may lead to forensic reservations.

In case of an HIV infection of the male partner, infectious viral particles can be separated from motile spermatozoa by means of a sequency of density gradient centrifugation, washing and swim-up (Fig. 1): HIV pregenomes and viruses can be detected in seminal plasma, the accompanying nucleate cell fraction and occasionally in immobile spermatozoa (Semprini *et al.*, 1992). Viable, motile spermatozoa are not considered to be virus carriers (review by Quayle *et al.*, 1998).

To exclude a contamination with viral particles with the greatest possible certainty, one aliquot of each prepared sample should then be tested for HIV nucleic acid by highly sensitive detection methods. Most commonly, each prepared sperm sample must be cryopreserved until all the test results are available. Prepared, HIV-negative spermatozoa can be used for all techniques of assisted reproduction in the healthy female partner, such as intrauterine insemination, conventional *in vitro* fertilization and intracytoplasmic sperm injection.

The couple has to be appropriately informed that even the most careful preparation techniques and testing methods cannot exclude the possibility of transmission to the partner (and thereby the child) with absolute certainty. By adhering to the described procedures and their comprehensive documentation, there should be no legal (liability-based) objections in performing assisted reproduction, wherever the male partner is infected with HIV. As a matter of principle, all female

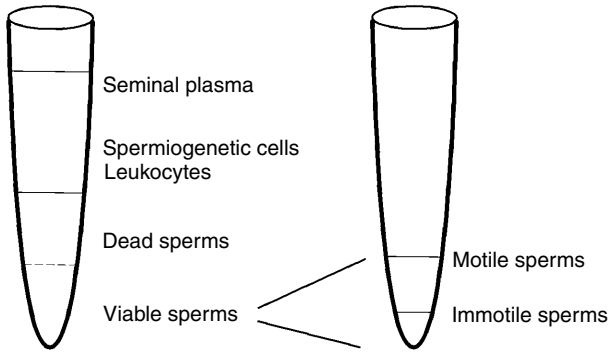

Figure 1. Sperm separation density gradient and swim-up.

patients undergoing one of the above procedures as well as any children born as a result, should be closely monitored for any change in infection status.

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